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Chemistry and Cytotoxicity

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The objective of the research has been to develop methods to separate, identify, and measure volatile and non-volatile compounds found in secondary wastewater effluent, and to test the suitability of the cytotoxicological assay for the substances found. Identification and measurement of volatile organics were achieved, and known substances were submitted for toxicological testing. Non-volatile substances were concentrated and fractionated and submitted for both toxicological and Ames mutagenicity testing. Toxicity testing utilized the effect of the fractions on both metabolic and bactericidal cellular activity. The use of platelets proved to be the most suitable because of their stability and correlation with gross human toxicity rankings. The less polar and non-polar fractions produced toxic responses in both metabolic and bactericidal assays. An initial set of samples submitted for mutagenicity testing showed definite activity in the more polar fractions. A second series of samples submitted for confirmatory testing showed no activity, indicating that the concentration of mutagens varies significantly with time.

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HEALTH EFFECTS OF CONSUMPTION OF RENOVATED WATER: CHEMISTRY AND CYTOTOXICITY

by

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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati (HERL) is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical or biological agents found in the environment. In addition to valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low-level insults.

This report presents data on the identification and measurement of organics in renovated wastewater along with development of toxicity testing techniques. These techniques could have application with direct and indirect reuse of municipal wastewater for potable purposes. With a better understanding of the degree of insult in our drinking water, measures may be developed to overcome some of these potentially harmful materials.

RJ Garner

R. J. Garner
Director
Health Effects Research Laboratory

ABSTRACT

The objective of the research has been to develop methods to separate, identify, and measure volatile and non-volatile compounds found in secondary wastewater effluent, and to test the suitability of the cytotoxicological assay for the substances found. Identification and measurement of volatile organics were achieved, and known substances were submitted for toxicological testing. Non-volatile substances were concentrated and fractionated and submitted for both toxicological and Ames mutagenicity testing. Toxicity testing utilized the effect of the fractions on both metabolic and bactericidal cellular activity. The use of platelets proved to be the most suitable because of their stability and correlation with gross human toxicity rankings. The less polar and non-polar fractions produced toxic responses in both metabolic and bactericidal assays. An initial set of samples submitted for mutagenicity testing showed definite activity in the more polar fractions. A second series of samples submitted for confirmatory testing showed no activity, indicating that the concentration of mutagens varies significantly with time.

This report was submitted by the University of Colorado in fulfillment of Grant No. R803968, under the sponsorship of the U.S. Environmental Protection Agency.

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SECTION 1

INTRODUCTION

The increasing demands placed on water supplies by an expanding population, industry, agriculture, and energy production have led to a growing interest in the potential for direct reuse of wastewater for human consumption. This interest is particularly intense in those areas of the world where water is already in short supply. At the same time there is considerable concern about the possible risks to human health of such a practice. It is difficult to assess these risks because of the large number of possible contaminants that might be present and because of the relative paucity of data about the effects of such contaminants.

For the past three years a group of researchers at the University of Colorado has been conducting research directed at developing better methods for establishing both the chemical identity and the biological effects of contaminants in both untreated and treated effluent from wastewater treatment plants. Most of this effort has been directed at the effluent from the Denver Metro Sewage Plant because the Denver Water Board is engaged in a multi-year effort to evaluate the feasibility of potable reuse of wastewater.

This research project has been a part of the Environmental Trace Substance Research Program (ETSRP) at the University of Colorado. The individuals involved in the program were:

Dr. Willard R. Chappell
Professor of Physics
University of Colorado at Denver
Professor of Preventive Medicine
University of Colorado Medical Center
Director, ETSRP

Dr. Harold Walton
Professor of Chemistry
University of Colorado at Boulder

Dr. Clive Solomons
Professor of Orthopedics
University of Colorado Medical Center

Dr. William Weston, M.D.
Associate Professor of Dermatology
University of Colorado Medical Center

During the first year of the project Dr. James Humbert was involved. When Dr. Humbert left the University of Colorado he was replaced by Dr. Weston. Dr. Elias Balbinder of the American Medical Center Cancer Research Center and Hospital in Denver has also been associated with the project. Dr. Balbinder has performed the Ames tests on some concentrates of Denver Metro sewage effluent, supplied by Dr. Walton.

The overall objectives of the research were:

1) To develop various analytical methods to concentrate, fractionate, and identify compounds, particularly those that proved to be toxic to the cells used.
2) To test the applicability of certain mammalian cells for use as in vitro models to evaluate the toxicity of wastewater.

The results of this work have several potential beneficial uses including:

1) The eventual development of methods for automatic monitoring.
2) Methods development for evaluating the human health effects of potable use.
3) The setting of guidelines for further toxicological testing and epidemiological studies.
4) The identification of the most important pollutants and classes of pollutants.
5) The development of gross tests for groups of contaminants.

The samples of wastewater and treated water were obtained by Dr. Walton and his students who then used various methods for concentrating and fractionating the samples. The concentrates and fractionates were then split, with samples being sent to Drs. Solomons, Weston, and Balbinder for biological testing, while Dr. Walton and his students performed chemical analyses.

The following sections describe the results of the research during the past three years. A very small fraction of the total effort was devoted to mutagenicity testing. Nevertheless, some important results were obtained and these are described in Appendix A. A Ph.D. thesis entitled Dynamic Headspace Enrichment in Trace Volatile Organic Analysis of Aqueous Environmental Samples, by Gary A. Eiceman, was also a product of this work.

SECTION 2

CONCLUSIONS

Considerable progress was made in the development of methods for the concentration, fractionation, and identification of organic compounds in treated and untreated water and wastewater. This work is described in detail in Section 3 mentioned earlier. Some of the principal results and conclusions were:

- The development of modifications to the "purge-and-trap" method that led to a significant simplification of the method. The method allows for measurements of volatile compounds at concentrations of one part per billion or less.

- A start was made on the more difficult problem of analyzing non-volatile compounds in aqueous solution. This method fractionates the compounds by polarity and concentrates them by as much as forty thousand times. A few weakly-polar compounds, present in concentrations below 100 parts per billion, have been identified.

- A significant result of our work was the demonstration of the presence of toluene, ethyl benzene, and the xylenes. They have appeared in all the wastewater samples and their concentrations are higher in chlorinated than in unchlorinated wastewater. It appears that they are produced from something in the wastewater, such as humic materials, by chlorination, yet we have chlorinated humic acid preparation in the laboratory and have found no toluene.

A considerable effort was made in the development and refinement of methods for in vitro toxicity testing. This was accompanied by the testing of concentrates and fractionates of water and wastewater. These results are described in detail in Sections 4 and 5. Some of the principal results and conclusions were:

- Considerable progress was made in the measurement of adenine nucleotides (ATP, ADP, and AMP). This approach allows the determination of the effect of contaminants on the energy metabolism of various cells. The use of a High Performance Liquid Chromatography (HPLC) significantly shortened the time required per determination to fifteen minutes per sample.

- Three human cells were studied: platelets, neutrophils, and monocytes. Platelets proved to be the most suitable for a continuous surveillance because they are more stable and the test requires smaller quantities of blood.

- In addition to the use of energy metabolism as a parameter, the effect of toxic substances on the phagocytosis and killing power of neutrophils and monocytes was studied.

- The neutrophil system was found to be relatively insensitive. Moreover, this system is difficult to work with and is very slow. It is not a suitable system for on-line monitoring.

- Monocytes are more stable than neutrophils and large batches can be processed at one time. The depression in monocyte function induced by known amounts of toxic compounds corresponds to the changes in the energy metabolism of the cells. However, the energy metabolism test is more sensitive than the bactericidal tests.

- Another test that was studied involved the antibody dependent cellular cytotoxicity (ADCC) assay. This measures the ability to eliminate cancer cells and terminate viral infections. This test proved to be relatively sensitive. Again, however, the monocytes are less stable than platelets and not as suitable for an online system.

- Several toxic compounds were tested by the various assays. The greatest sensitivity was found to be with the platelet metabolism which showed a good correspondence with the relative gross toxicity (e.g. LD50 for rats).

Numerous samples of water and wastewater (both treated and untreated) were collected from various sources. The results of the various chemical and biological tests on these samples are described in detail in the text. Some of the principal results and conclusions were:

- When wastewater is subjected to the fractionation method several distinct fractions were obtained. These fractions differ in polarity. The biological testing showed that most of the toxicity was at the non-polar end of the gradient. This toxicity was reflected in both the metabolic and bactericidal assays.

- Mutagenic activity was tested by the Ames assay. Definite mutagenic activity has been observed in the more polar fractions. However, this activity is not always present, which suggests that the concentrations of the responsible mutagens varies with time.

- Chemical analysis indicated that reverse osmosis allowed some material to pass that was weakly polar or non-polar.

- One fraction that is obtained from wastewater is of particular interest because it has at times been both mutagenic and toxic. It is a green, polar material. This green material has appeared in every wastewater sample we have tested. Its visible absorption spectrum shows a sharp peak at 630 nm. Gel permeation chromatography shows it to contain at least two substances, a green component of low molecular weight and a pale brown component of high molecular weight.

- Humic material spreads itself across the gradient, thus complicating chemical analyses.

- A number of compounds have been identified in several wastewaters including chloroform, 1,1,1-trichloroethane, benzene, ethyl benzene, and chlorotoluene.

- While most of the biological assays were responsive to varying degrees in tests involving concentrates and fractions of wastewater, the metabolic assay using platelets and the Ames test seems to provide the most suitable tests because of speed, ease, reliability, and correspondence with gross toxicity and carcinogenicity.

SECTION 3

CHEMICAL ANALYSIS

INTRODUCTION

It is convenient to group the organic constituents of natural water, treated water and wastewater into volatile and non-volatile compounds. Much attention has been given to the volatile compounds, for they are easily separated and identified, yet they represent only a small fraction of the total organic content. The non-volatile compounds are much more difficult to identify. Indeed, the characterization of non-volatile organic constituents of water and wastewater is one of the most difficult and intractable problems facing the analytical chemist today.

To study the volatile impurities we used the "purge-and-trap" method, developed by Bellar and Lichtenberg (1), Bertsch (2,3), Grob (4), May (5) and others, with modifications of our own that are described in detail in the thesis of G.A. Eiceman (6). For the non-volatile impurities we have turned to the "trace-enrichment" technique of Little and Fallick (7), Creed (8) and others; this technique is described below.

In this investigation we collaborated closely with the Denver Water Board, and in particular with the staff of the Metropolitan Denver Sewage Treatment Plant, where we collected most of our wastewater samples. The Columbine Water Treatment Plant at Thornton, a few kilometers down the South Platte River from the Denver Sewage Treatment Plant, was the source of some samples, as were the sewage plants of Boulder and the Upper Big Thompson Sanitary District plant at Estes Park. We also received two air-freight shipments of wastewater from the personnel of the San Jose Creek Water Quality Laboratory of Los Angeles County, California. These samples were taken at the Pomona, California wastewater treatment plant. One sample was taken from the secondary clarifier effluent, another from a location following activated carbon treatment, and a third after final chlorination of the carbon-treated effluent.

Other samples of water from springs, rivers and lakes were taken locally. In this report we shall describe the principal analytical results, while placing most emphasis on methodology.

VOLATILES

The "Purge-and-Trap" Method: Outline of Procedure

Our method was adapted from that of May, et al. (5) and is similar to that of Bertsch (2,3). Purified nitrogen gas was passed through a one-liter sample of water to be analyzed, which was contained in a 2-liter three-neck flask mounted on a heating mantle. One "neck" carried a thermometer. Nitrogen was led into the flask through a tube that terminated in a glass frit; it passed out of the flask into a glass tube or trap, 175 mm long and 4 mm internal diameter, packed with 150 mg of the adsorbent Tenax-GC, a porous polymer of phenylene oxide. This adsorbent strongly retains hydrocarbons and chlorinated hydrocarbons while having little affinity for water. The trap was surrounded by a water jacket and could be cooled.

After loading with volatile organic compounds from the sample, the Tenax trap was removed and mounted at the inlet of a gas chromatograph, which was fitted with a wall-coated capillary column and a flame-ionization detector. The adsorbed organic compounds were transferred from the trap to the capillary column by first cooling the column in dry ice, then quickly heating the trap by mounting a two-piece oven around it. Nitrogen was passed; it swept the organic compounds out of the trap and into the column, where they were "focused" close to the column entrance. The column oven was then turned on, evaporating the dry ice and raising the temperature to 180° in 3 hours according to a linear program. The FID response was recorded. Peaks were identified by mass spectrometry and by comparison of retention times with those of known compounds.

Gas Chromatography Details

The chromatograph was a Varian Model 2400 with modifications to be described. The column was a 30-meter glass capillary, 0.25 mm internal diameter, 0.5 mm outer diameter, drawn in a Hupe capillary drawer. It was coated by the following procedure: A 1% aqueous solution of benzyl triphenylphosphonium chloride was passed, then the column was baked at 200° for one hour with nitrogen passing. Rinsing and baking were repeated twice, then a 5% solution of silicone oil OV-101 in toluene was passed, and the toluene evaporated at room temperature. The column was mounted in the chromatograph and conditioned by passing nitrogen at 200° for one hour.

The chromatograph was arranged as shown in Figures 1 and 2. To transfer the volatile organic compounds from the trap to the column, some 500 g of dry ice were placed in the column chamber, out of contact with the column. The trap, unheated, was connected as shown in Figure 1, with the 4-port valve in the "by-pass" position, so that the nitrogen carrier gas flowed

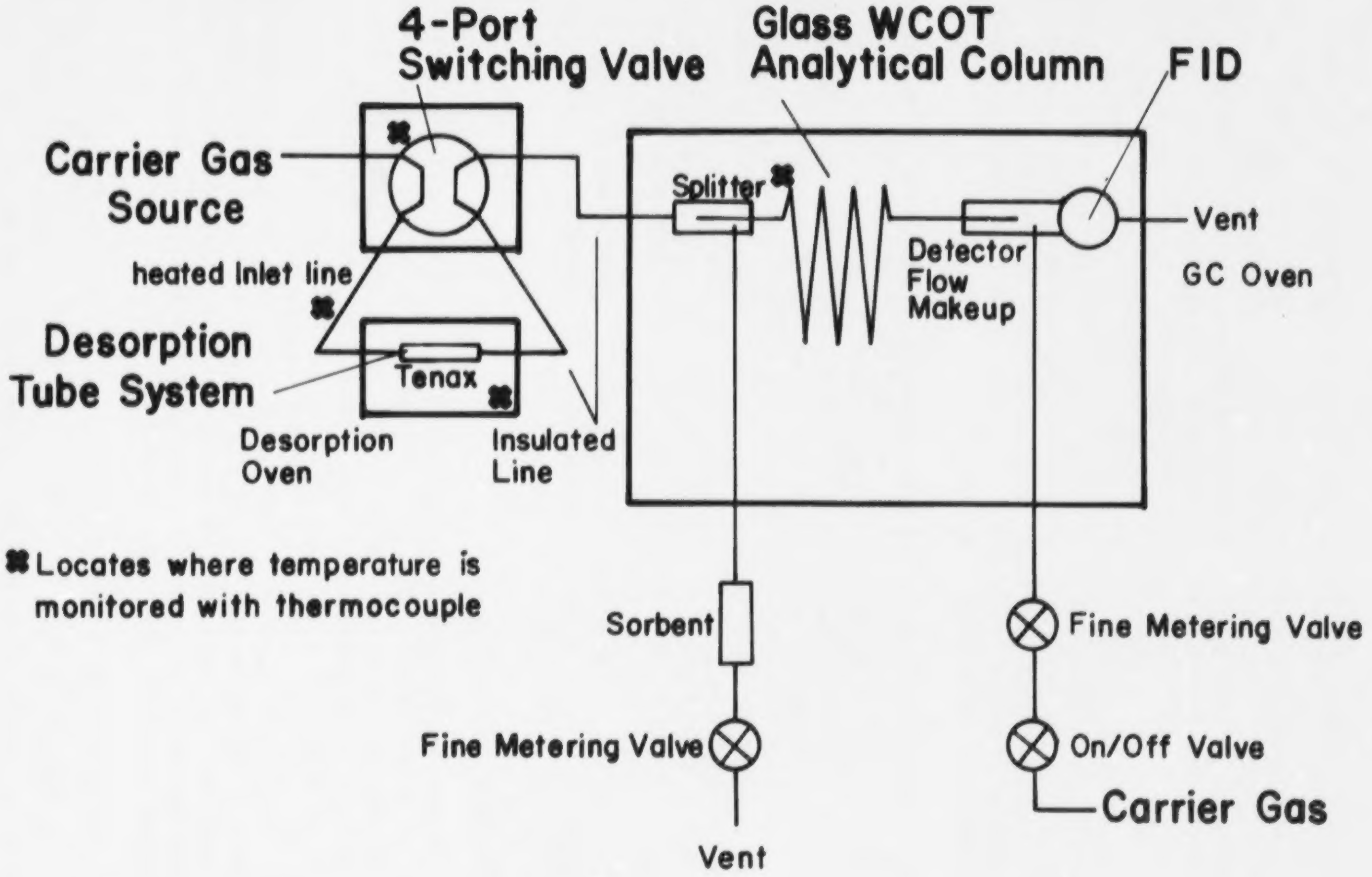

4-Port
Switching Valve
Glass WCOT
Analytical Column
FID
Carrier Gas
Source
heated Inlet line
Desorption
Tube System
Tenax
Desorption
Oven
Insulated
Line
Splitter
Detector
Flow
Makeup
Vent
GC Oven
✱ Locates where temperature is
monitored with thermocouple
Sorbent
Fine Metering Valve
Vent
Fine Metering Valve
On/Off Valve
Carrier Gas

Figure 1. Gas chromatographic assembly.

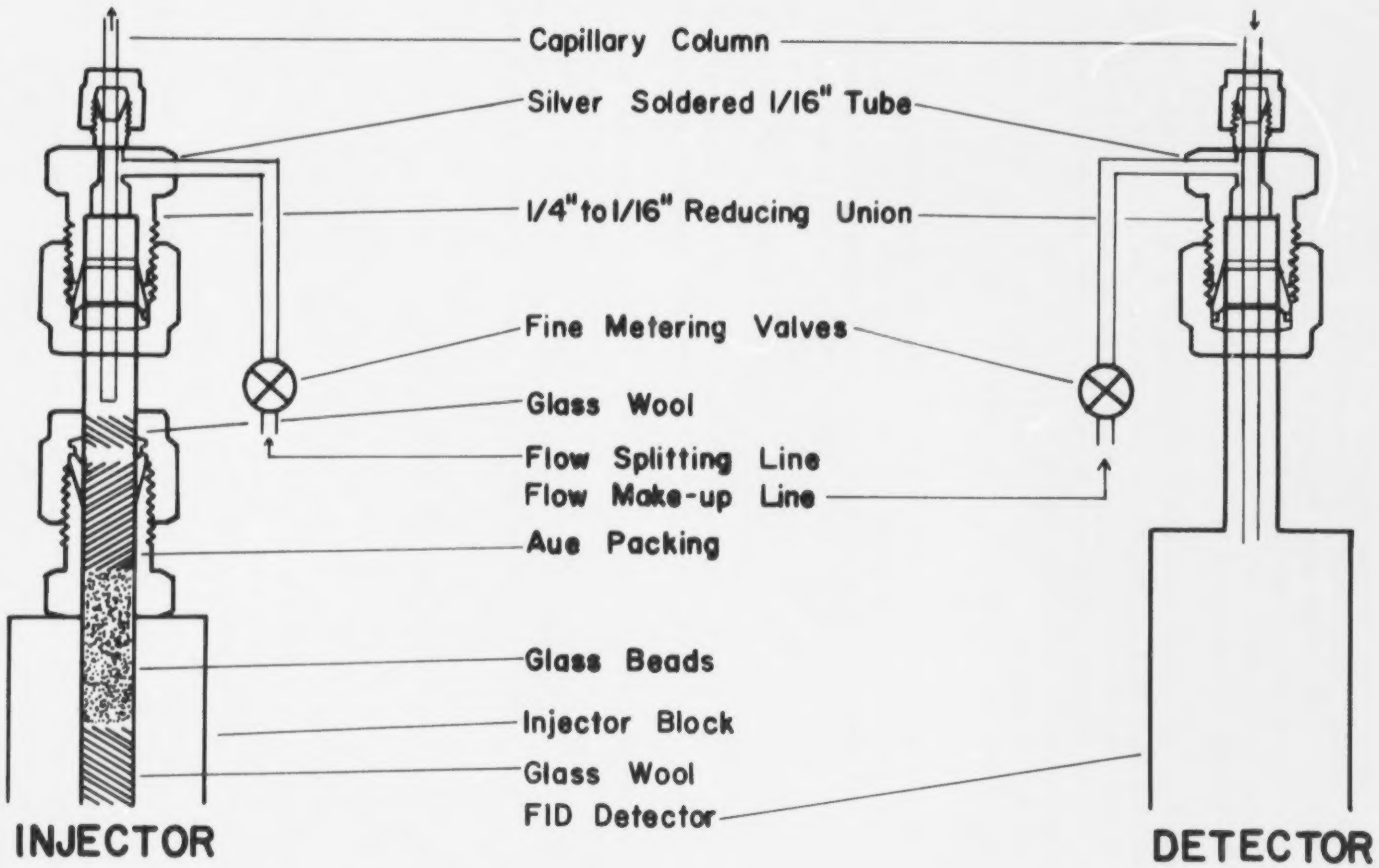

Capillary Column
Silver Soldered 1/16" Tube
1/4" to 1/16" Reducing Union
Fine Metering Valves
Glass Wool
Flow Splitting Line
Flow Make-up Line
Aue Packing
Glass Beads
Injector Block
Glass Wool
FID Detector
INJECTOR
DETECTOR

Figure 2. Details of injector splitter and connection to flame-ionization detector.

directly into the column. When the column temperature had fallen to -40°, the 4-port valve was turned to the "load" position and the two-piece desorption oven, preheated to 185°, was placed around the trap. The trap was mounted so that the nitrogen flowed in the opposite direction to the flow during purging. After 5 minutes the valve was again turned to "by-pass" and the column temperature program started.

A splitter was used to avoid overloading the column; the splitting ratio was 4:1. Ahead of the splitter was an injector unit (Figure 2), whose function was to screen out accidental particles and to ensure thorough mixing of the entering gases. It also permitted one to make direct injections with a syringe for calibration purposes. At the exit of the capillary column nitrogen was added at 30 mℓ/min as make-up for the flame-ionization detector.

For calibration, known amounts of known compounds were introduced. They were introduced in one of three ways: (a) by direct introduction of a solution of the compound in carbon disulfide; (b) by first loading a Tenax trap with a solution of the compound in methanol, passing nitrogen for 2 minutes at room temperature to remove the methanol, then attaching the trap to the injection port and proceeding as just described; or (c) by preparing one-liter portions of solutions of known concentrations in pure water, then sparging (purging) in the manner described.

To make these solutions, very pure water was required. It was made from the house distilled water by first passing it through a large column of the macroporous polystyrene resin Amberlite XAD-2 (cleaned by refluxing with methanol), then redistilling. The redistilled water gave good blanks (see Figure 3).

Retention Times and Retention Efficiencies

Table 1 shows retention times, and programmed retention indices referred to *n*-alkanes. The retention indices are more reproducible than the times themselves. Peak heights were proportional to mass, between 0.5 and 5.0 μg of the compounds tested; with loadings greater than 5 μg it was best to use peak areas for quantitative measurement.

The effectiveness of retention on the Tenax trap was measured by first introducing 0.5 μg of a compound directly into the chromatograph, then loading a Tenax trap with another 0.5 microgram portion and desorbing the compound into the chromatograph (procedures (a) and (b) above). The percent recoveries of 17 compounds, with standard deviations, are listed in Table 2. They are essentially quantitative.

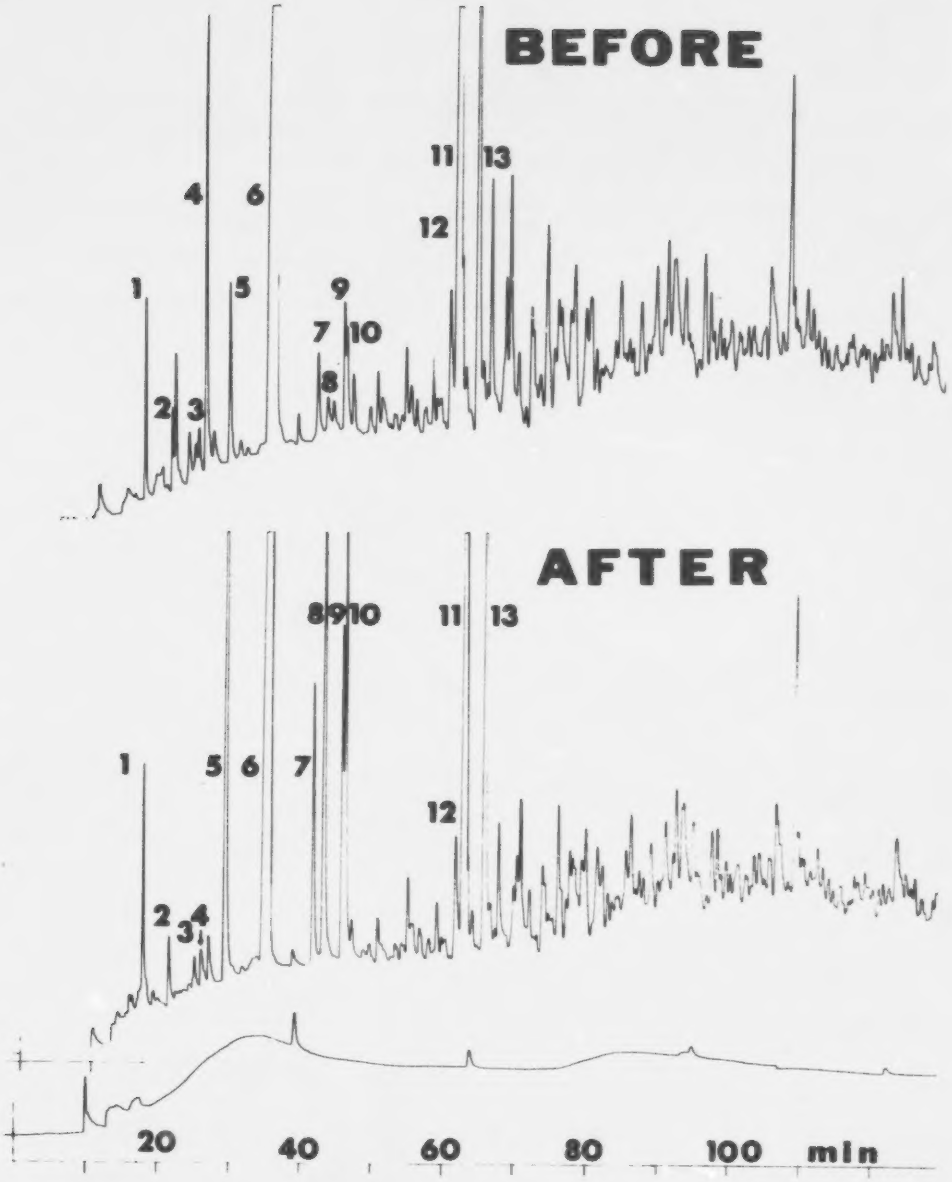


Figure 3. Chromatogram of Denver secondary effluent (a) before, and (b) after chlorination; ordinates are on FID response. (1) chloroform (2) 1,1,1-trichloroethane (3) trichloroethylene (4) dimethyldisulfide (5) toluene (6) tetrachloroethylene (7) p- and m-xylene (8) ethlybenzene (9) styrene (10) o-xylene (11,12,13) p-,m-,o-dichlorobenzene.

TABLE 1. RETENTION TIMES, PROGRAMMED RETENTION INDICES AND STANDARD DEVIATIONS (IN PARENTHESES) FOR A WALL-COATED OPEN TUBULAR COLUMN

Compound	Time, Min.	Retention Index
n-Hexane	9.4 (0.8)	---
n-Heptane	17.8 (1.0)	---
n-Octane	30.7 (1.0)	---
n-Nonane	44.7 (1.7)	---
n-Decane	60.9 (1.8)	---
n-Dodecane	93.8 (0.6)	---
1,2-Dichloroethane	11.2 (0.7)	620 (7.0)
Benzene	12.5 (0.8)	637 (1.5)
Trichloroethylene	16.1 (0.8)	678 (1.1)
Bromotrichloromethane	22.1 (0.7)	733 (1.6)
Toluene	23.6 (0.7)	745 (1.6)
Tetrachloroethylene	29.3 (0.9)	789 (1.0)
Chlorobenzene	33.5 (1.1)	819 (0.5)
p-Xylene	36.3 (1.3)	839 (0.8)
Ethyl benzene	37.7 (1.6)	850 (0.8)
o-Xylene	40.4 (1.5)	869 (0.6)
p-Chlorotoluene	49.3 (1.9)	928 (1.1)
m-Dichlorobenzene	56.5 (1.9)	972 (1.1)

TABLE 2. PERCENT RECOVERY OF 500 NANOGRAMS FROM SPIKED TENAX TRAP BY THERMAL DESORPTION AT 185°C (RELATIVE STANDARD DEVIATION IN PARENTHESES)

n-Heptane	78	(10)
n-Octane	89	(31)
n-Nonane	109	(24)
n-Decane	74	(68)
n-Undecane	115	(37)
n-Dodecane	107	(32)
Chloroform	93	(40)
1,2-Dichloroethane	108	(45)
Bromotrichloromethane	83	(19)
Toluene	99	(20)
Tetrachloroethylene	99	(23)
Chlorobenzene	119	(38)
para-Xylene	114	(46)
Ethylbenzene	117	(36)
ortho-Xylene	113	(41)
para-Chlorotoluene	135	(10)
meta-Dichlorobenzene	150	(16)

Purging Parameters and Efficiencies

The limiting factor in the purge-and-trap method is the transfer of compounds from the water sample to the Tenax trap. Once on the trap they can be efficiently desorbed. In order to get them on to the trap, they must first be swept out of the water, then adsorbed by the Tenax in competition with water vapor, then retained while the purge gas is passing. To measure the overall efficiency of recovery we added 0.5-microgram quantities of 20 compounds to separate one-liter portions of water, then went through the purging and trapping procedure, varying the conditions and comparing the recoveries. The conditions were varied as follows.

Purging Time--

With the solution at 70° and the trap cooled to 10°, and a nitrogen flow of 150 mℓ/min., purging times from 15 to 120 minutes were tried. With *n*-pentane and chloroform the recoveries in 15 minutes were 2% and 8% respectively; they dropped to zero with 60 minutes purging. Evidently they are adsorbed so weakly that prolonged nitrogen flow sweeps them out of the trap. Benzene and trichloroethylene gave best recoveries (46% and 36%, respectively) with 30 minutes purging. All other compounds gave recoveries that increased as purging time increased. The difficult step is to get these compounds out of the water. Once in the gas stream they are efficiently adsorbed.

Gas Flow--

Nitrogen was passed for 120 minutes at flow rates between 75 and 300 mℓ/min. With all compounds but the least volatile (chlorobenzene, *m*-dichlorobenzene and *p*-chlorotoluene) recoveries were best at the slowest flow rate. If the nitrogen was only passed for 20 minutes, however, faster flow gave better recovery. For 1,2-dichlorethane, bromotrichloromethane and benzene, 100 mℓ/min was better than 250 mℓ/min. Faster flow gave better purging but poorer trapping.

Trap Temperature--

Thinking that by cooling the trap the adsorption of water by the Tenax might interfere with the retention of organic compounds, we made tests with the trap cooled to 10° by the water jacket and other tests in which the trap was allowed to come to ambient temperature. The recoveries of heptane, nonane, chloroform, trichloroethylene, bromotrichloromethane, tetrachloroethylene, toluene and chlorobenzene were better with the trap at ambient temperature. In view of the experience of other workers, however (4), it may be better to cool the trap.

Purging Temperature--

It was established that the sample solutions should be heated above room temperature while purging. The temperature of 70° seems to be the best. At higher temperatures the purge gas carries too much water vapor.

Trap Size--

Tests were made with traps containing 50 and 100 mg Tenax; recoveries were not as good as with 150 mg. Putting two 150-milligram traps in series we found that the most volatile compounds, chloroform, 1,2-dichloroethane, trichloroethylene and benzene, would break through the first trap and be adsorbed on the second. For most compounds one 150-milligram trap was sufficient.

The best conditions for purging and trapping depend on the compound sought. We adopted the following procedure as a compromise:

Purge the sample with nitrogen at 250 mℓ/min for 20 minutes, starting with the sample at 10° - 20°, and setting the heating mantle so that the temperature rises to 70° within the 20 minute period. Do not cool the trap. After purging, remove the trap and dry it by passing nitrogen at room temperature for 5 minutes at 50 mℓ/min., then transfer it to the gas chromatograph. Recoveries obtained by this procedure are listed in Table 3.

Environmental Samples

Wastewater--

Figure 3 shows chromatograms obtained from the secondary effluent of the Metropolitan Denver sewage plant, before and after chlorination, along with a system blank obtained with redistilled water. The chlorinated effluent is discharged into the river. Peaks are seen for the following compounds, listed in the order of emergence from the capillary column, with the most prominent peaks indicated by asterisks:

*Chloroform
1,1,1-Trichloroethane
Benzene
Dibromomethane
Trichloroethylene
*Dimethyldisulfide
*Toluene
**Tetrachloroethylene
*Xylenes
Ethyl benzene
Styrene
Methyl ethyl benzenes
Trimethyl benzenes
**Dichlorobenzenes
Chlorotoluenes

Figure 3 shows an effect that we have observed repeatedly, namely, an increase in the toluene and xylene concentrations after chlorination (see also Figure 7). The cause of this increase is unknown. We have tried to produce it in the laboratory by treating sewage effluents by chlorine and by chlorinating solutions of humic acids, with no success. Table 4, however, suggests that the effect is real. This table shows the concentrations of toluene and other compounds found at various stages of treatment in the Columbine water-treatment plant, Thornton,

TABLE 3. RECOVERIES FROM AQUEOUS SOLUTION BY PURGE-AND-TRAP METHOD (PERCENT; RELATIVE STANDARD DEVIATIONS IN PARENTHESES)

Compound	Concentration in μg/ℓ 0.5	 2.5	 5.0
n-Pentane	0	0	8 (45)
n-Heptane	66 (20)	56 (20)	70 (10)
n-Octane	81 (58)	45 (80)	27 (26)
n-Decane	59 (54)	37 (80)	17 (20)
Chloroform	15 (28)	12 (20)	38 (10)
1,2-Dichloroethane	12 (70)	23 (20)	53 (14)
Benzene	>45	37 (15)	68 (24)
Trichloroethylene	>40	62 (9)	82 (4)
Bromotrichloromethane	11 (50)	18 (27)	46 (28)
Toluene	130 (60)	50 (29)	85 (38)
Tetrachloroethylene	120 (35)	83 (25)	89 (10)
Chlorobenzene	60 (50)	60 (16)	54 (13)
p-Xylene	83 (12)	80 (10)	67 (10)
Ethyl benzene	75 (13)	75 (10)	67 (10)
p-Chlorotoluene	65 (10)	66 (10)	70 (6)
m-Dichlorobenzene	67 (28)	61 (10)	64 (7)

TABLE 4. ESTIMATED CONCENTRATIONS OF VOLATILE ORGANICS IDENTIFIED IN COLUMBINE WATER TREATMENT PLANT THORNTON, COLORADO

Treatment stage date	Concentration in μg/ℓ				
	Chloroform	Toluene	Bromodichloromethane	Tetrachloroethylene	p-,m-Xylene
11/30/77					
Raw	ND	0.1	ND	0.3	ND
Pretreatment	ND	0.1	ND	0.1	ND
Clarifier*	0.5	0.3	0.6	0.1	0.2
Final	0.7	0.5	0.8	0.3	0.2
12/7/77					
Raw	0.01	0.1	ND	0.03	ND
Pretreatment	0.01	0.02	ND	0.1	0.6
Clarifier*	0.1	0.1	0.09	0.2	0.03
Final	0.1	0.2	0.1	0.2	0.08
12/13/77					
Raw	0.2	0.08	ND	0.2	0.05
Pretreatment*	0.1	0.2	0.6	0.2	0.05
Clarifier	0.7	0.5	0.7	0.2	0.08
Filter	1.0	0.5	0.8	0.2	0.08
Final	0.07	0.3	0.7	0.2	0.05

*Site of chlorination

ND is not detected, estimated lower limit of detection is 1-10 ng/ℓ

Colorado. (The city of Thornton draws some of its drinking water from shallow wells near the South Platte River, a few kilometers downstream from the discharge of the Metropolitan Denver sewage treatment plant, and the pattern of volatile organic compounds shows that the well water is very similar to that of the Denver effluent).

Other investigators have noted concentrations of benzene, toluene and xylenes in the range 0.1-0.4 μg/ℓ in finished drinking waters that were not present in the raw waters (9).

Chromatograms of the first shipment of Pomona wastewater, in March 1978, are shown in Figures 4 and 5. Figure 4 shows the secondary effluent before carbon treatment. Figure 5 shows the wastewater after carbon treatment. Peaks due to toluene and xylenes are prominent, though they appear to be reduced by carbon treatment, relative to the chlorinated aliphatics. Figure 6 shows chromatograms of all three stages in the Pomona wastewater shipped in May. Here we can see that carbon treatment reduced the concentrations of aromatic compounds, at least with respect to the chlorinated aliphatics, chloroform and trichloroethane.

At the Upper Big Thompson Sanitary District plant near Estes Park, Colorado, the secondary treated sewage effluent is being sterilized by ozone before discharging into the river. Figure 7 shows chromatograms of this water before treatment at the plant, as well as after treatments with ozone and chlorine in the laboratory. The difference in the effects of ozone and chlorine treatments is very clear. Ozonation produces n-alkanes and aldehydes (10). Chlorination produces chlorotoluenes and, apparently, toluene itself. The toluene peak is prominent in all chromatograms except that of the undisinfected water.

River waters, potable waters, spring and lake waters--

A typical polluted river is the South Platte, which flows through Denver. A chromatogram of the volatile compounds upstream from the sewage plant is shown in Figure 8 and one taken after the river has received the sewage plant effluent plus the water of highly-polluted Sand Creek is shown in Figure 9. The contrast is obvious. The concentrations found from the chromatograms, after allowing for the flame-ionization response factors and overall recovery efficiencies (Table 3), are shown in Table 5.

Below the Denver sewage plant, on the South Platte River, is the city of Thornton. As we have mentioned, Thornton draws some of its drinking water from shallow wells near the river. A chromatogram of one of these wells appears in Figure 10, together with chromatograms of the river water and of the Denver sewage effluent. The persistence of the peaks due to dichlorobenzenes, in particular, indicates that traces of organic compounds from Denver enter the Thornton well.

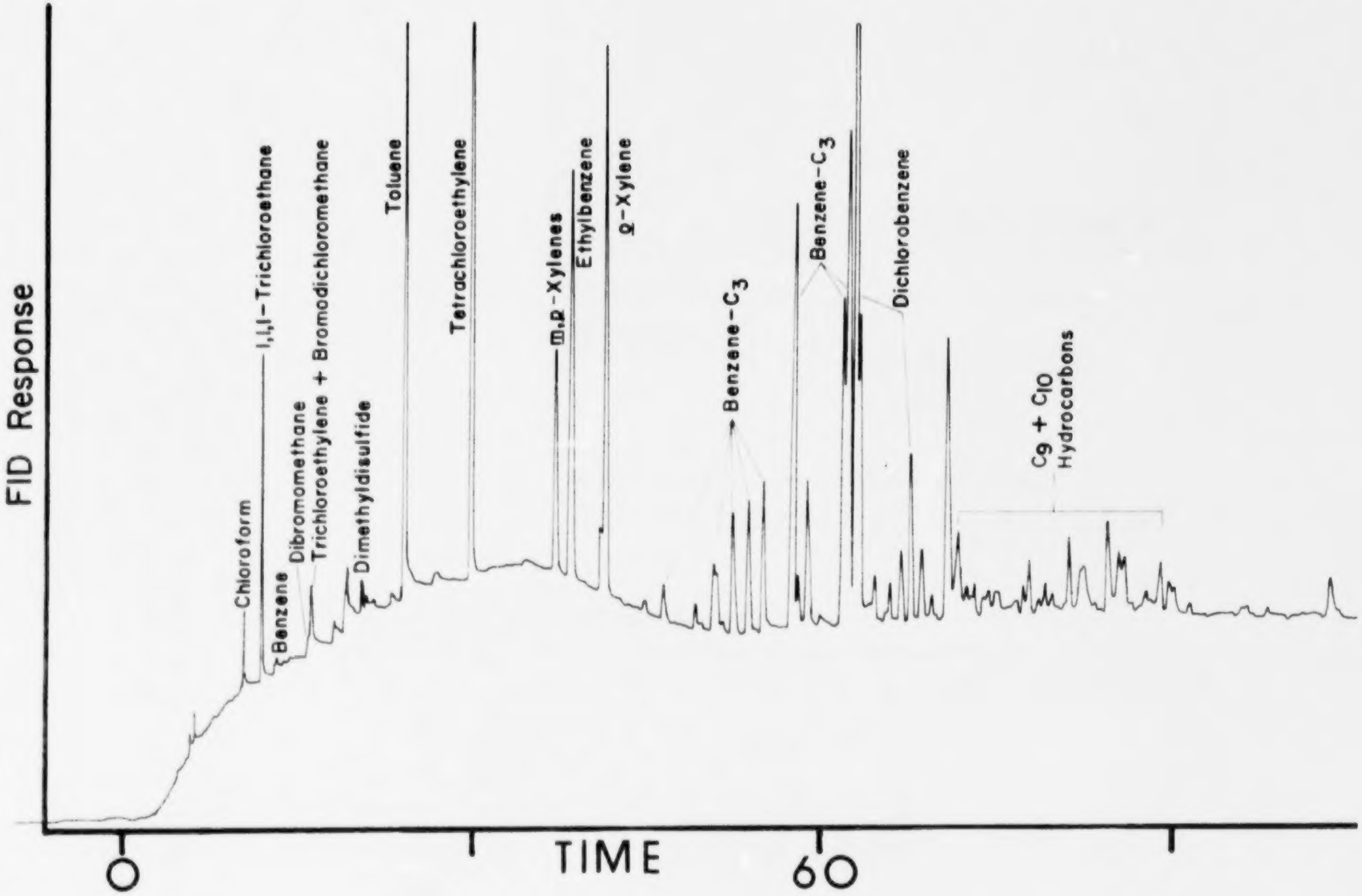


Figure 4. Chromatogram of secondary clarifier effluent, Pomona, California wastewater plant, March, 1978.

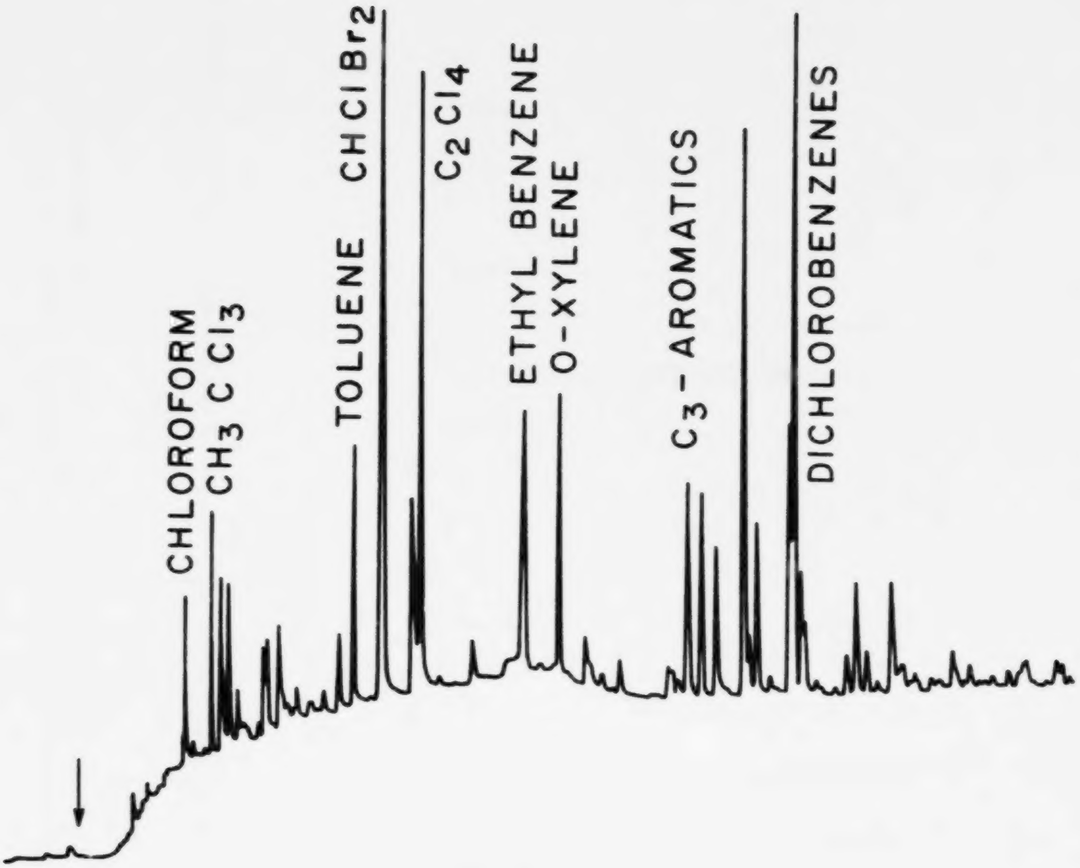


Figure 5. Chromatogram of Pomona, California wastewater following carbon treatment, March, 1978.

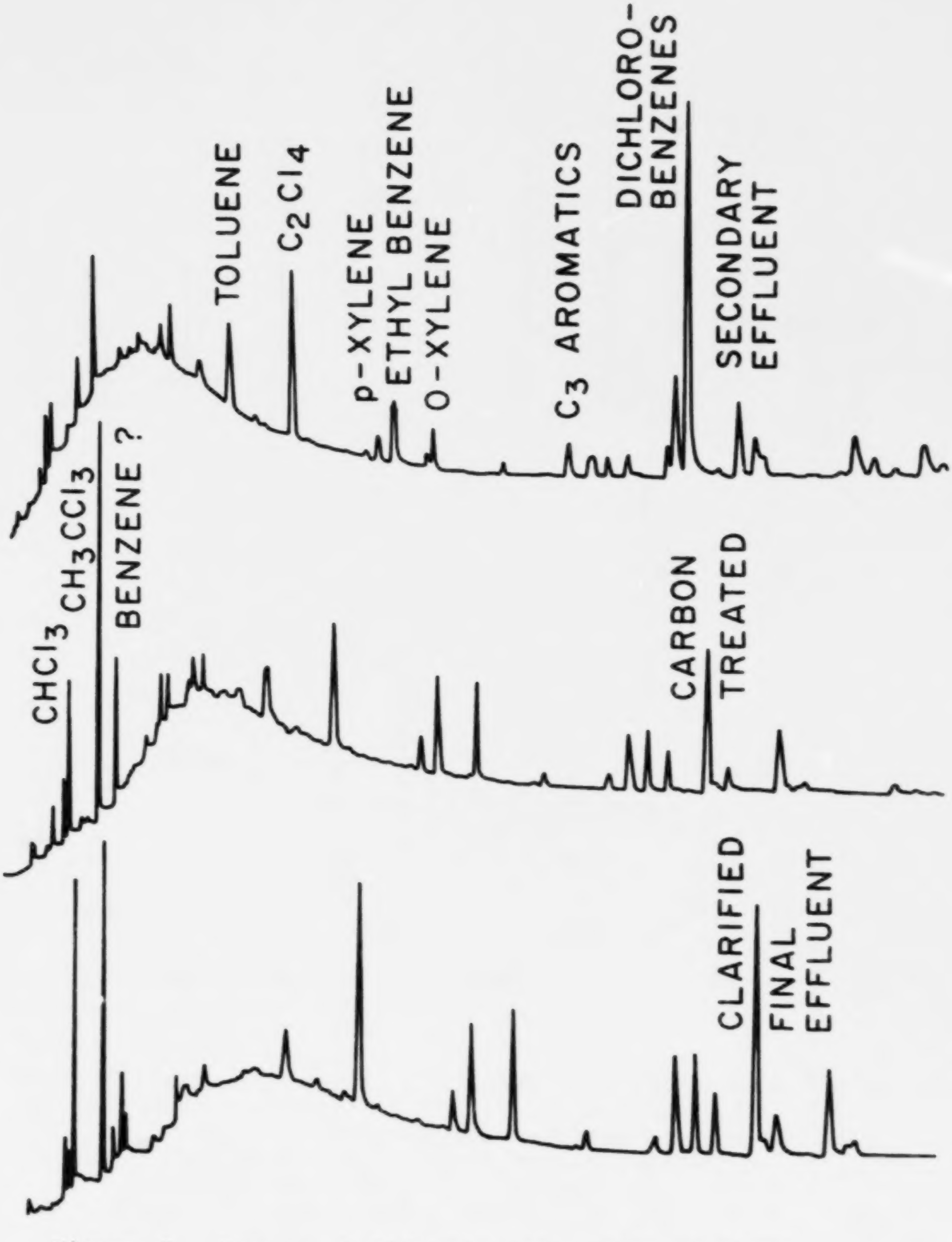


Figure 6. Pomona, California wastewater at three stages of treatment, May, 1978.

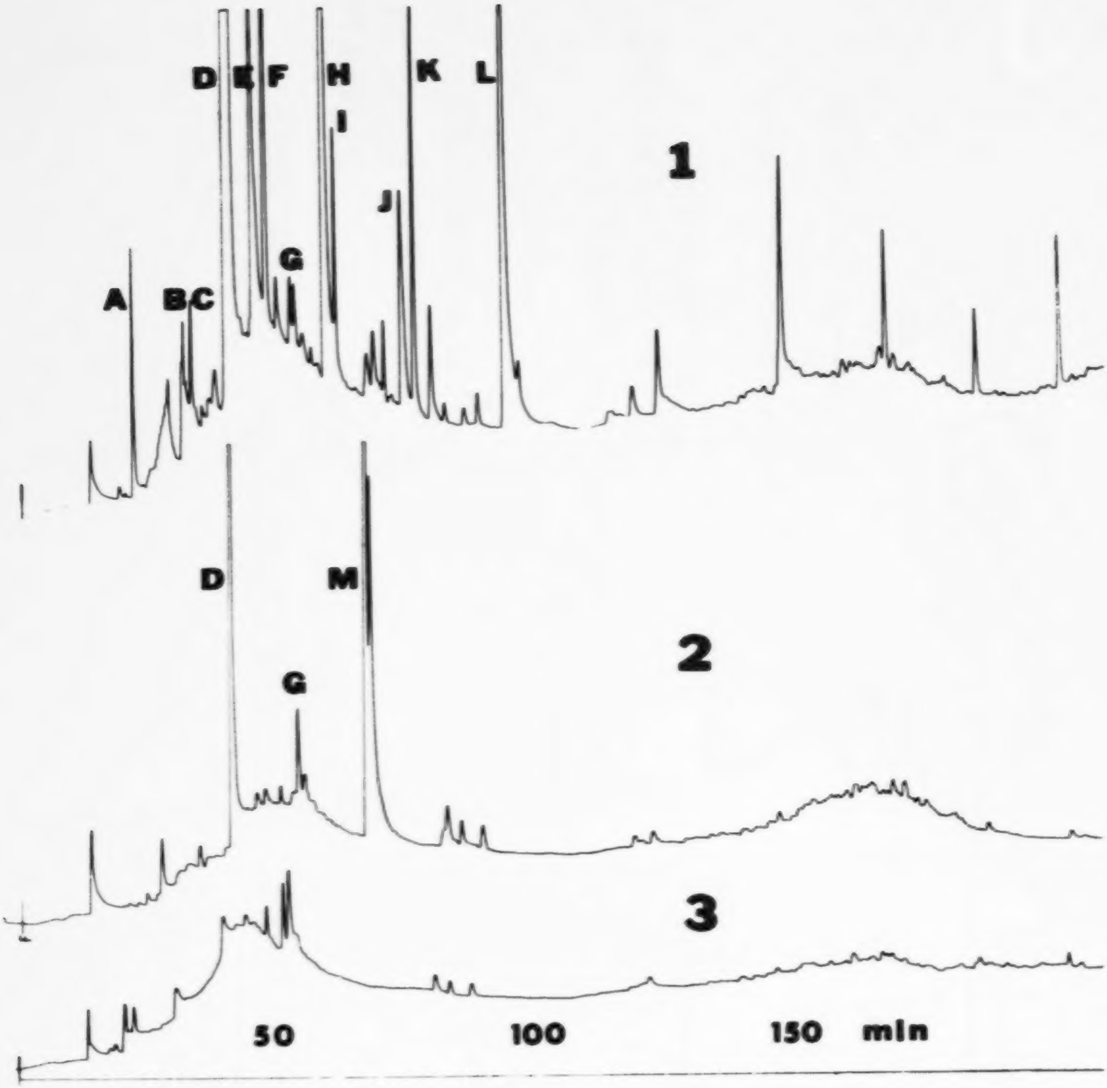


Figure 7. Wastewater from Upper Big Thompson plant: (1) after ozone treatment; (2) after chlorine treatment; (3) secondary effluent before sterilization.
(A) hexane (B) pentanal (C) heptane (D) toluene (E) hexanal (F) octane (G) xylenes (H) heptanal (I) nonane (J) octanal (K) decane (L) nonanal (M) chlorotoluenes.

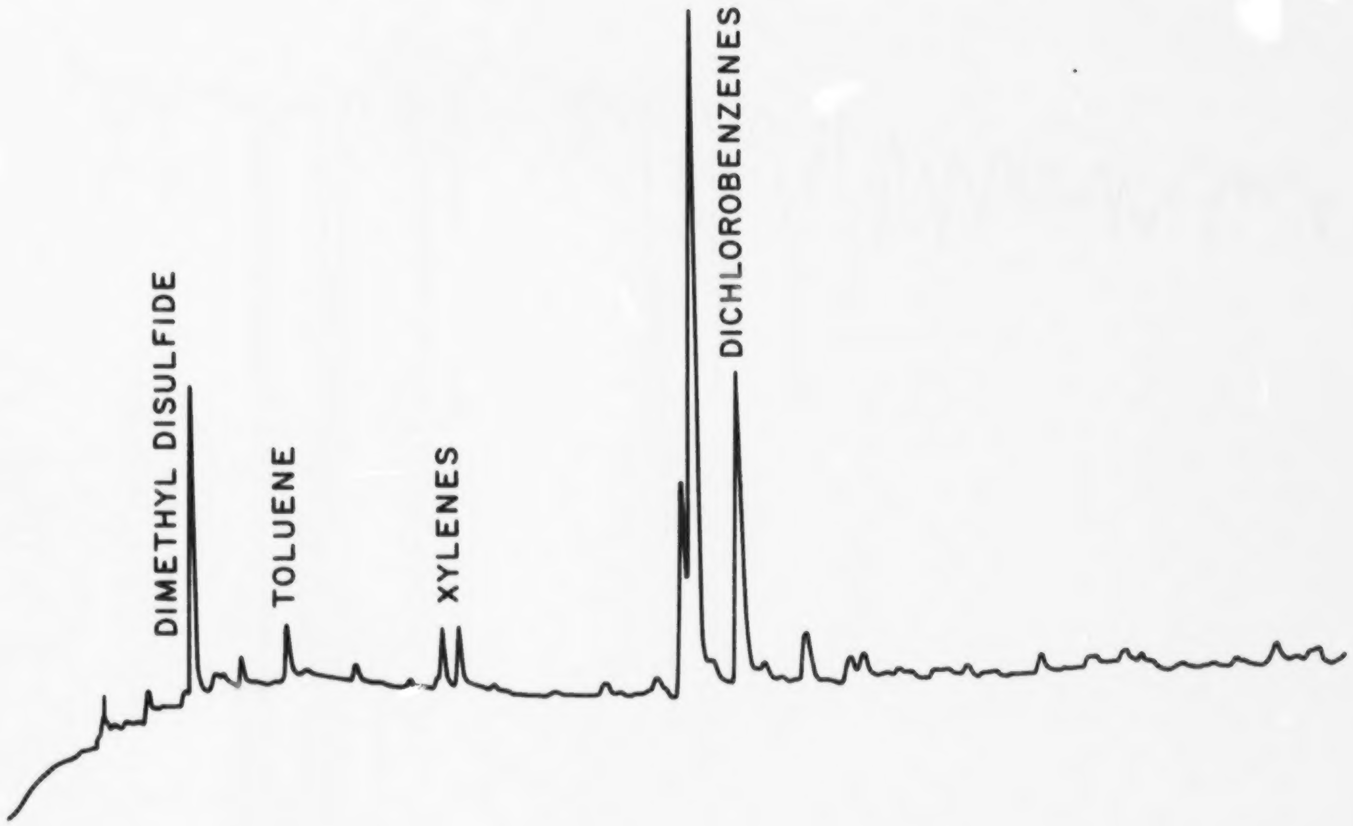


Figure 8. South Platte River, above sewage plant.

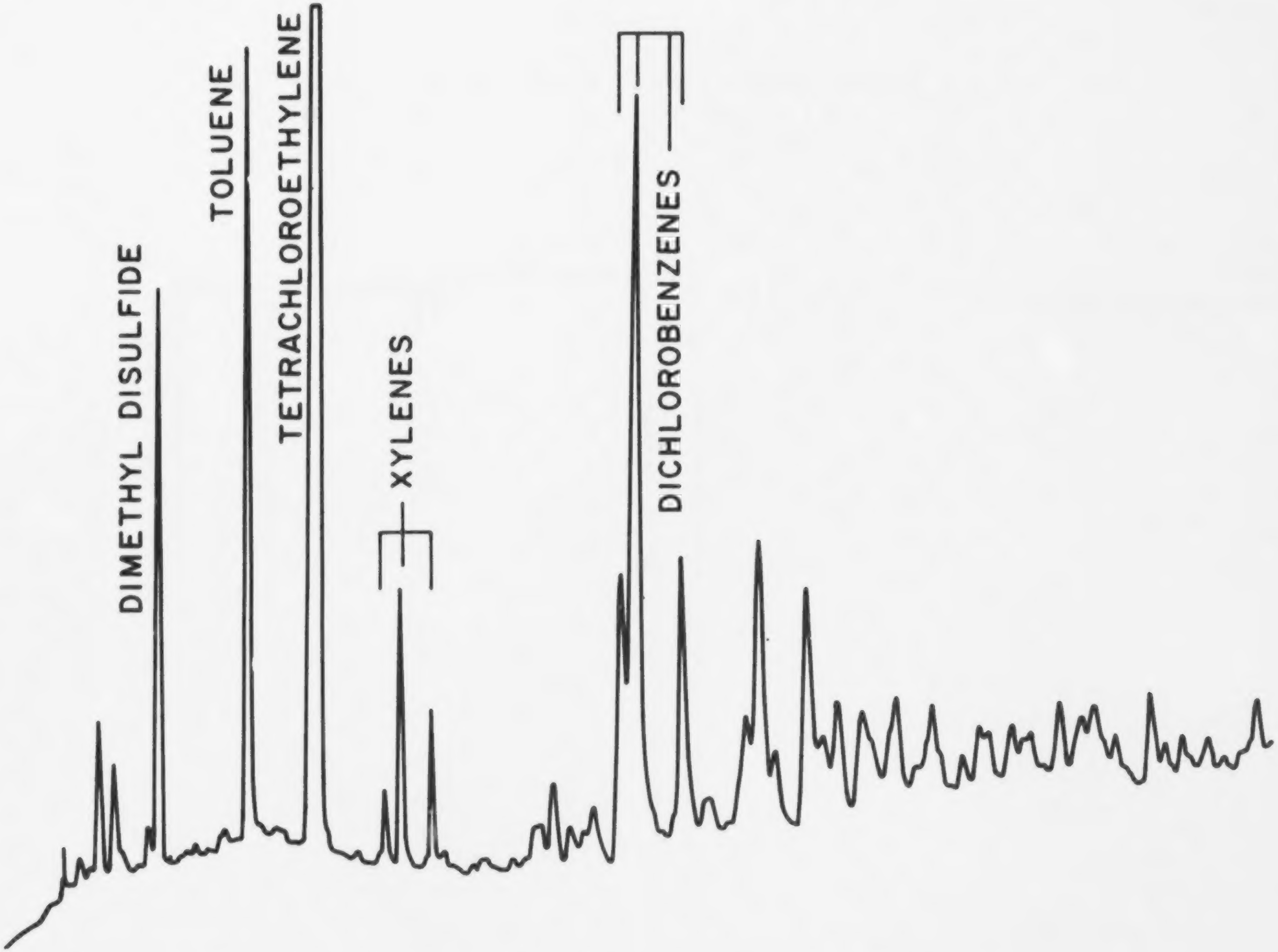


Figure 9. South Platte River below sewage plant and confluence with Sand Creek.

TABLE 5. CONCENTRATIONS OF VOLATILE ORGANICS IN DENVER-AREA WATERS AND WASTEWATERS, IN MICROGRAMS PER LITER (ND=NOT DETECTED)

Sample	Chloroform	Toluene	C_2Cl_4	Xylenes, o-,m-,p-	Dichlorobenzenes o-,m-,p-,
South Platte, upstream of sewage	ND	2	3	0.1,0.5,0.2	0.2,1,0.9
South Platte, downstream of sewage	4	3	18	0.2,0.7,0.4	3,9,3
Denver sewage effluent	0.2	1	40	0.05,0.1,0.05	5,23,6
Thornton Well (downstream from Denver)	2	0.2	0.2	0.2,0.2,0.1	3,8,4

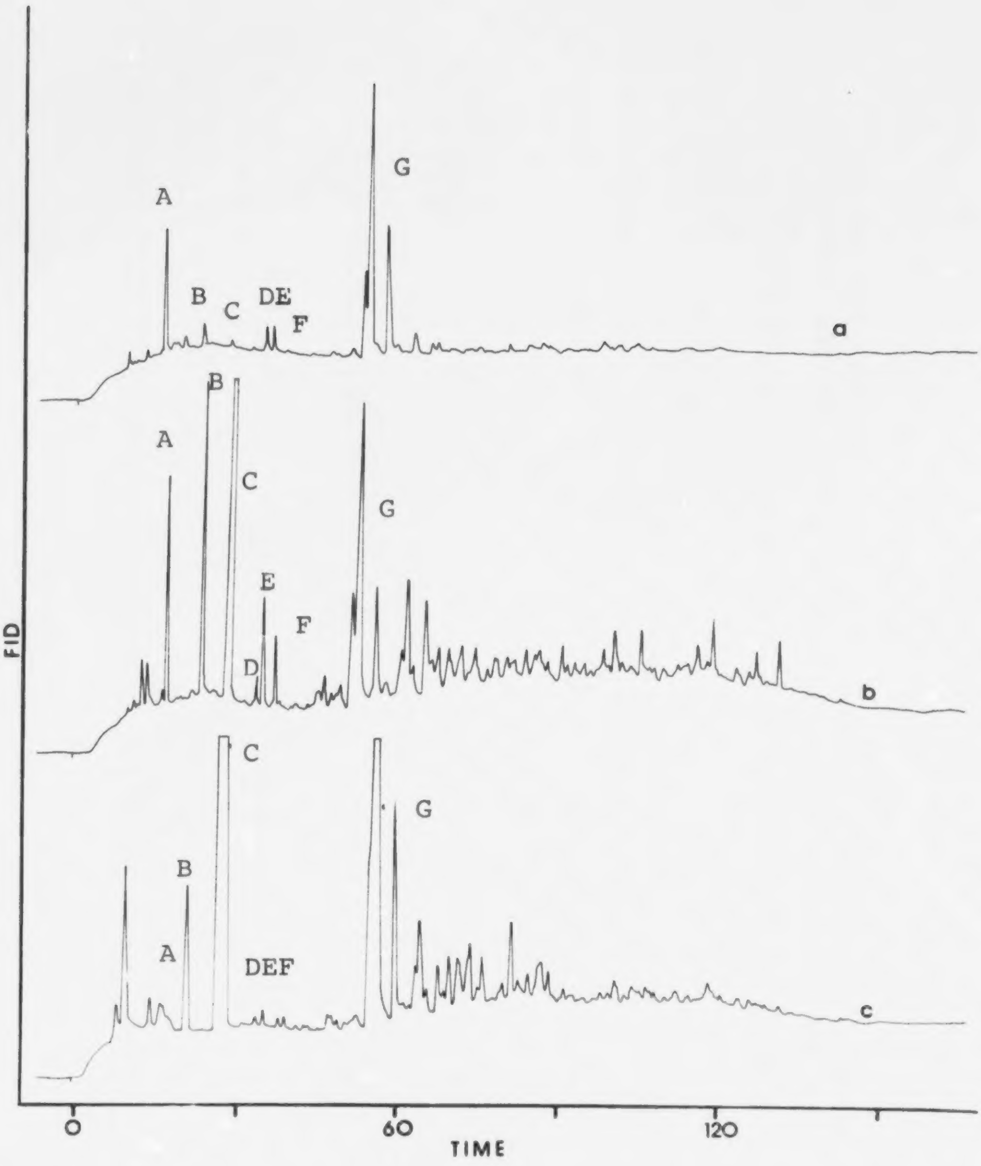


Figure 10. Volatile organics in water from (a) Thornton well, 3-1, (b) South Platte River near well, (c) Denver sewage plant effluent. Peaks identified: (A) chloroform (B) toluene (C) tetrachloroethylene (D) m-and p-xylene (E) ethyl benzene (F) o-xylene (G) dichlorobenzene.

Water from an experimental reverse osmosis unit at the Denver sewage plant showed marked peaks at positions corresponding to chloroform, toluene and tetrachloroethylene, as well as lesser peaks, indicating that reverse osmosis did not remove the more volatile compounds. Activated carbon treatment does not remove these compounds either. Liquid chromatography, however, showed that carbon filtration and reverse osmosis were effective in removing the less volatile and more polar impurities from water (see below).

Tap waters from Denver and Boulder were examined and showed very little volatile content, well below 1 μg/ℓ. The city of Boulder, Colorado, has an unusually clean source of drinking water. Humic material is present, but there is little contamination of human origin. The water is chlorinated and clarified with alum before distribution. No toluene or other aromatic hydrocarbons were found at any stage of the treatment, though trihalomethanes were found in low concentrations. It seems that precursors of toluene may exist in wastewater but not in uncontaminated natural waters.

Pristine mountain water from a spring in the timber at 8,000 feet elevation (2,400 meters) showed five peaks, two of high volatility emerging near the beginning of the temperature program, and three of low volatility emerging near the end. The concentrations were about 0.1 μg/ℓ. Identifications were not attempted, but one may speculate that the compounds came from the decay of humic material. Similar peaks of low volatility appeared in Denver tap water. Water from Grand Lake, Colorado was almost devoid of volatile organic matter.

NON-VOLATILE COMPOUNDS

Non-Volatiles by "Trace Enrichment"

The trace enrichment technique depends on the absorptive properties of porous silica coated with chemically-bonded octadecyl groups (C_{18}). This material is a universal absorbent for all organic compounds except those that are very water-soluble, ionized or highly polar, and it is widely used as a column packing in liquid chromatography. If water that contains traces of dissolved organic compounds is pumped through a tube or column packed with octadecyl silica, the less polar compounds are retained. Even phenol and caffeine, compounds that dissolve significantly in water and are considered to be fairly polar, are held quite strongly. One can pump a large volume of a very dilute solution, of concentration 1 ppm or less, and find that these compounds are quantitatively retained.

The absorbed compounds can be released and stripped from the column by passing a water-miscible organic solvent like methanol or acetonitrile. Mixtures of the solvents with water may be used, and a selective release of the absorbed substances is accomplished by adding methanol to water in progressively higher proportions, starting with pure water and ending with pure methanol, according to a solvent program or gradient. The most water-soluble or polar compounds leave the column first, and the most hydrophobic or non-polar compounds leave last. Each compound or group of compounds can be recovered as a relatively concentrated solution, and the concentration can be further raised by evaporating the solvent. Organic compounds originally present in, say, five liters of water can be concentrated into volumes of 2 mℓ or less.

The apparatus we use is shown in Figure 11. The pumps are Waters Assoicates Model 6000 A adjustable-speed liquid chromatography pumps; they are controlled by a Waters Associates Model 600 Solvent Programmer. The column is of stainless steel, 50 cm long, 1 cm internal diameter. Following the column is an ultraviolet liquid chromatography detector; normally this was a single-wavelength detector reading the absorbance at 254 nm, though a Schoeffel variable-wavelength detector was sometimes used. The signal from the detector was recorded. A typical record obtained with wastewater and most city water supplies is shown in Figure 12. The significance of the record is discussed in the next section.

The column was packed with Bondapak C_{18}-Porasil B, from Waters Associates. This material has a large particle size, 37-75 μm, and is usually used for preparative chromatography. Its chromatographic resolving power is rather poor; it is rated by Waters Associates at 350 plates per meter. We chose it, rather than the high-resolution Micro-Bondapak C_{18}, because it absorbed and desorbed the constituents of wastewater reversibly and never clogged up. In our first summer's work we used expensive, prepacked, microparticulate, analytical columns and ruined two of them; an irreversible sorption took place after pumping several liters of water, the back pressure rose to an intolerable level, and we were not able to clean and unplug the columns. We have used the Bondapak C_{18}-Porasil B column for two years and it flows as easily as ever; the back pressure with water at 7 mℓ/min is about 600 psi. The brown humic materials of wastewater are reversibly absorbed and desorbed. After a year's operation we flushed the column with acetonitrile, chloroform and hexane, and extracted 100 mg of white, waxy solid whose infrared spectrum was that of a straight-chain paraffin with some -C-O- linkages. This was the only indication of irreversible absorption.

The Bondapak was packed dry into the column; no special equipment or technique was needed. Though the resolving power is modest, it is sufficient for our present needs. We tested other packings, including graphitized carbon and the porous polystyrene gel Hitachi-3010, and found them to retain humic materials irreversibly.

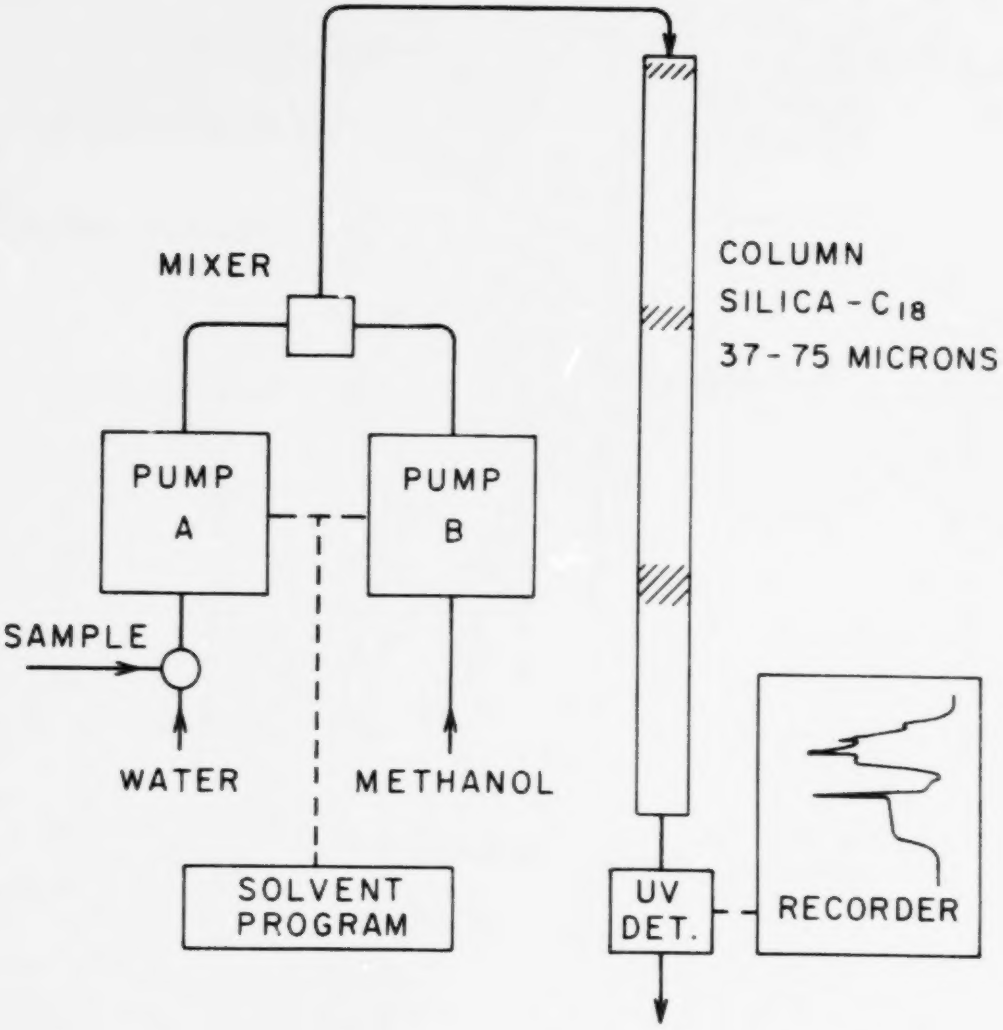

MIXER
COLUMN
SILICA-C_{18}
37-75 MICRONS
PUMP A
PUMP B
SAMPLE
WATER
METHANOL
SOLVENT PROGRAM
UV DET.
RECORDER

Figure 11. Apparatus for trace enrichment.

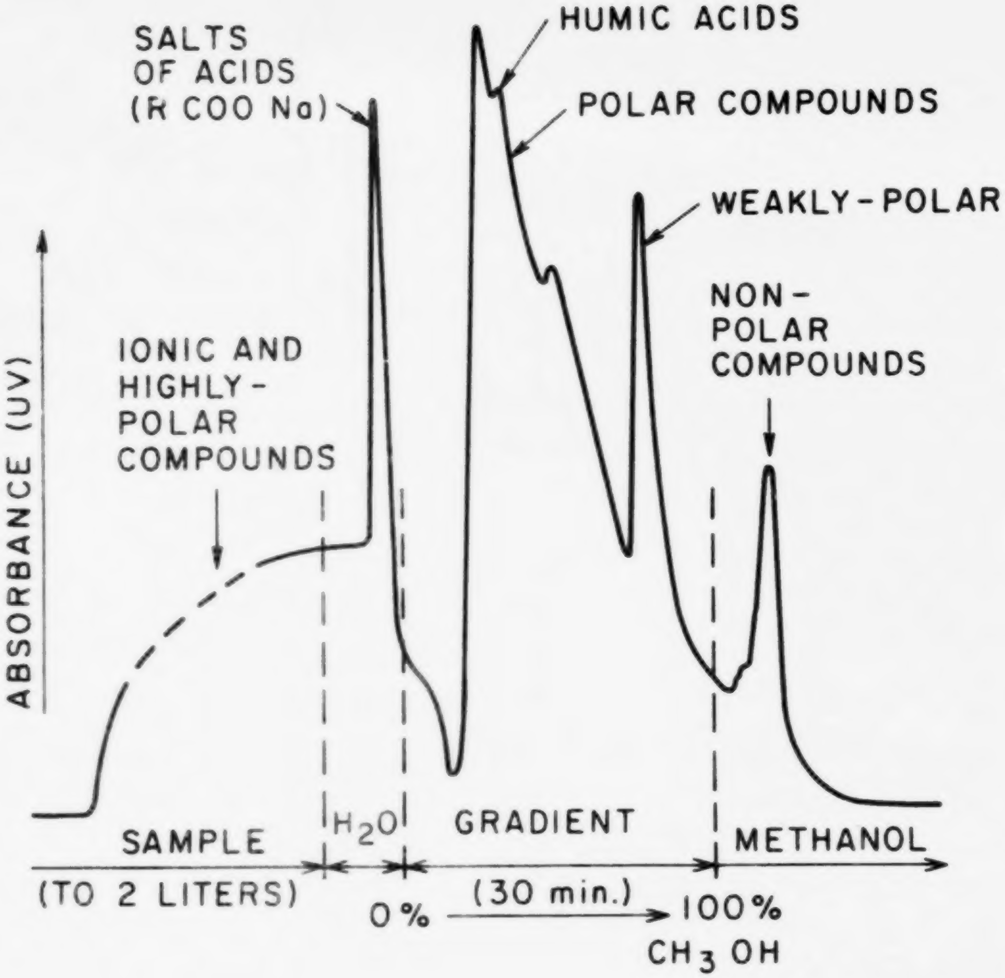


Figure 12. Chromatogram obtained in trace enrichment.

We went to the large-sized column (1 cm x 50 cm) to produce fractions that would be large enough for toxicity tests and for more detailed chemical examination. Our practice is to collect a relatively small number of fractions on the first operation, to test each fraction for toxicity and mutagenic action, and then to subdivide each fraction further, paying special attention to the more toxic fractions. The mode of operating the column is summarized here:

Sample Preparation--

Filter under suction through glass fiber filters, finishing with 0.7-micron filter. Before the last filtration, heat the sample to 70° - 80°; suction filtration then removes dissolved air. Finally, adjust the pH to 7.5 - 7.8 by adding nitric acid.

Filtration is necessary to avoid blocking the pumps or the column; dissolved air must be removed to avoid vapor locks in the pumps. The pH adjustment is needed to avoid chemical attack on the porous silica packing (which occurs above pH 8) and to convert the weak organic acids of the sample to their anionic forms. The effect of pH on the elution pattern will be described below.

In some experiments where we wanted to process a large amount of sample, we performed a preliminary concentration by fractional freezing, using a Model 3-1000 freeze concentrator (Virtis Co., Gardiner, NY). This operation was done at the Water Quality Laboratory of the U.S. Geological Survey in Lakewood, Colorado. The "freeze concentrate" was one-tenth the volume of the original wastewater and was difficult to filter because of the high concentration of detergents and suspended clay. Most of the suspended matter was removed by centrifugation, after which the filtering was relatively easy.

Sample Loading--

Pump the desired volume of filtered sample through the Bondapak column at 7 mℓ/min., using one Waters Model 6000 A pump. In the early work the volume was 2 liters of wastewater or 1 liter of 10:1 freeze concentrate. Later we routinely pumped 6-10 liters of filtered wastewater to get enough material for further study.

Another way to handle larger samples is to use an auxiliary "collector column" 15 cm x 1.0 cm internal diameter, packed with the same Bondapak-C_{18} that was used in the main analytical column. We pumped sewage effluent through this column without deaerating it or adjusting the pH; however, between the pump and the collector column were three tubes packed with filtering materials, glass beads, sand, and glass wool. The pump was a Madden diaphragm pump that was insensitive to suspended matter and could be left to run all day without attention. After pumping 3 gallons (12 liters) of secondary sewage effluent through the collector column at 0.5 liter per hour we disconnected the column, flushed it with distilled water, connected it to the inlet of the analytical column and started the water-to-methanol program that is described in the next section.

A collector column of Bondapak-C_{18} was used by May, et al. (5) to concentrate poorly-volatile hydrocarbons from sea water and marine sediments; they attached the loaded column to an analytical column of MicroBondapak C_{18} and applied a water-to-methanol gradient, as we have done. Use of a separate collector column is clearly more convenient than pumping the whole sample through the main column, but with wastewater, the suspended matter causes a serious problem. The "on-line" filters that we have used so far are inefficient. Our collector column became fouled with black material after a few gallons of wastewater had passed, and it lost its effectiveness. We shall experiment with better on-line filters to see if this fouling can be avoided.

Flushing, Gradient Elution--

With the solvent programmer activated and the methanol pump (B) (see Figure 11) ready to operate, reduce the flow rate to 5 mℓ/min and turn the solvent selector valve of the water pump (A) to admit pure water. (This was a house distilled water that had been passed through a bed of Amberlite XAD-2 resin and redistilled in glass.) Pump pure water for 15 minutes, then start the water-to-methanol gradient, a linear gradient from 0% to 100% methanol in 30 minutes. Meanwhile, run the UV detector and recorder, and collect samples as appropriate. We use the scheme shown in Figure 13.

Testing the Fractions--

Collect each fraction in a glass-stoppered bottle, and concentrate to the final volume desired (usually 2 mℓ) on a rotary evaporator (Buchi Rotavapor-M). Transfer the concentrated fractions to 4-milliliter glass vials for storage. In treating the last fractions (G5 in Figure 13), where the solvent is practically pure methanol, see if solids that stick to the glass evaporation flask separate and are not transferred to the sample vial. If this is happening, rinse the evaporation flask with small amounts of 95% ethanol to ensure that all solutes are removed and transferred. Note the approximate alcohol concentration for the information of those who will perform toxicity tests.

It is in the form of these 2-milliliter concentrates that the fractions are submitted for mutagenicity and toxicity testing. For chemical testing (secondary chromatography) we preferred to evaporate fractions G4 and G5 nearly to dryness and take up the residues in methanol.

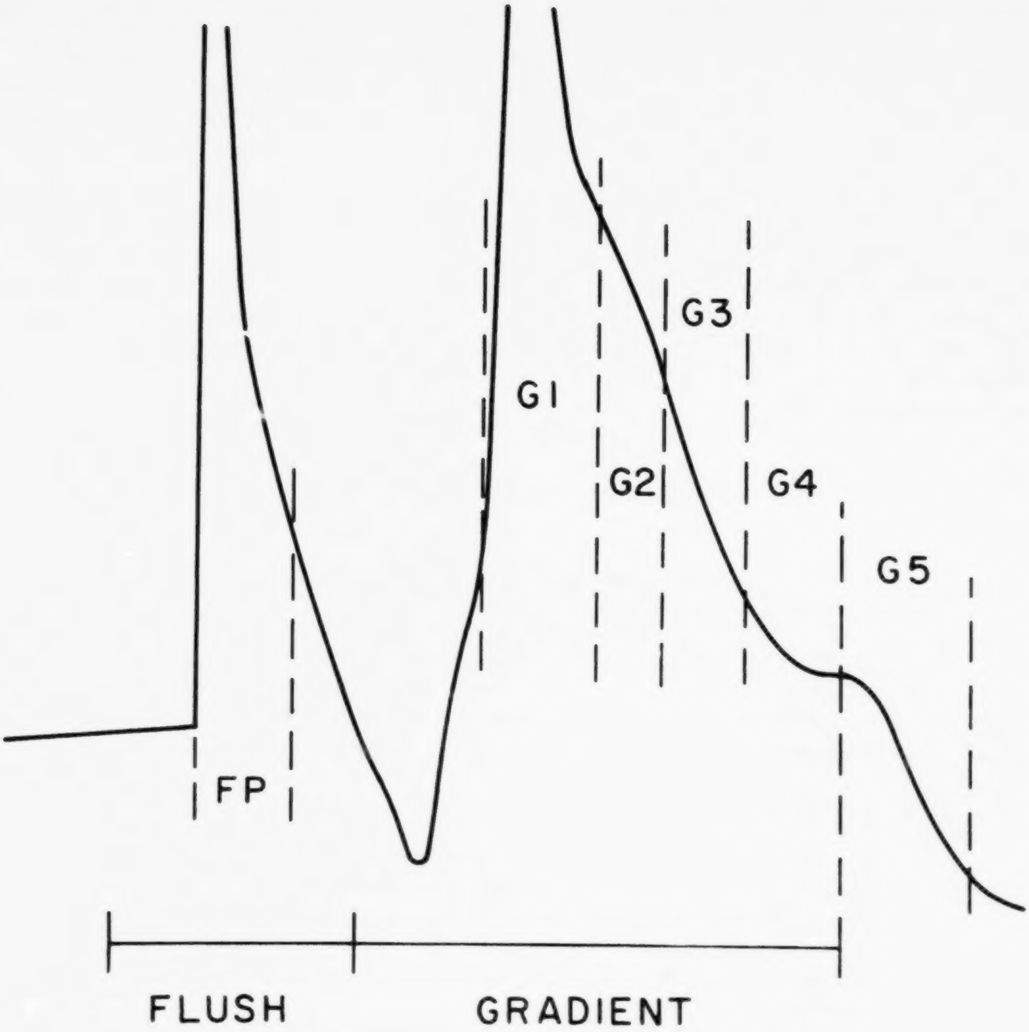


Figure 13. Trace enrichment chromatography (schematic) with system of fractions taken.

The Liquid Chromatography Elution Record

The chart-paper record of UV absorbance against time and solvent volume looks like Figure 12 (schematic) or Figure 14 (an actual wastewater). If the column is originally filled with pure water, and wastewater is then introduced, the UV absorbance rises sharply, as soon as the wastewater enters the detector, and eventually levels off. We see that some UV-active material passes through the column without being absorbed at all, while other material is held weakly by the column. The column is soon saturated with these weakly-absorbed substances.

The substances that pass through the column during loading account for some 60-75% of the total organic carbon in the sample. Total organic carbon was measured with a Dohrmann Envirotech Analyzer, Model DC 52-D. They do not appear to be toxic, and we have therefore not tried to characterize them, but we can expect them to be salts of relatively strong acids (like uric acid, $pK_a = 3.9$) and substances that are very soluble in water, like sugars, which were found in wastewater by Pitt and others (11). Sugars and many other organic compounds would not be "seen" in the ultraviolet. Inorganic salts likewise pass through the column without being retained or "seen" in the ultraviolet.

After the sample has been pumped we pass redistilled water for 15 minutes to flush the column before beginning the methanol gradient. As soon as the distilled water reaches the detector the UV absorbance rises abruptly to a high maximum, then falls more gradually. We call this peak of absorbance the "flush peak". We have found the following facts about the material in the "flush peak":

- It is moderately toxic
- Its pH is about 8
- Its UV spectrum is changed by adding acid, suggesting that salts of weak acids are present.
- The total organic carbon accounts for about 5% of the organic carbon originally in the wastewater.
- The peak height is decreased if the pH of the sample is lowered.
- Several substances are present, and they can be partially resolved by liquid chromatography on a column of anion-exchange resin. Some of the peaks from the resin column can be shown to be due to anions, others to neutral compounds. A refractive index detector placed in series with the ultraviolet detector reveals no new peaks; that is, the compounds of the flush peak are all aromatic in character.
- The solution flushed from wastewater is light brown in color.

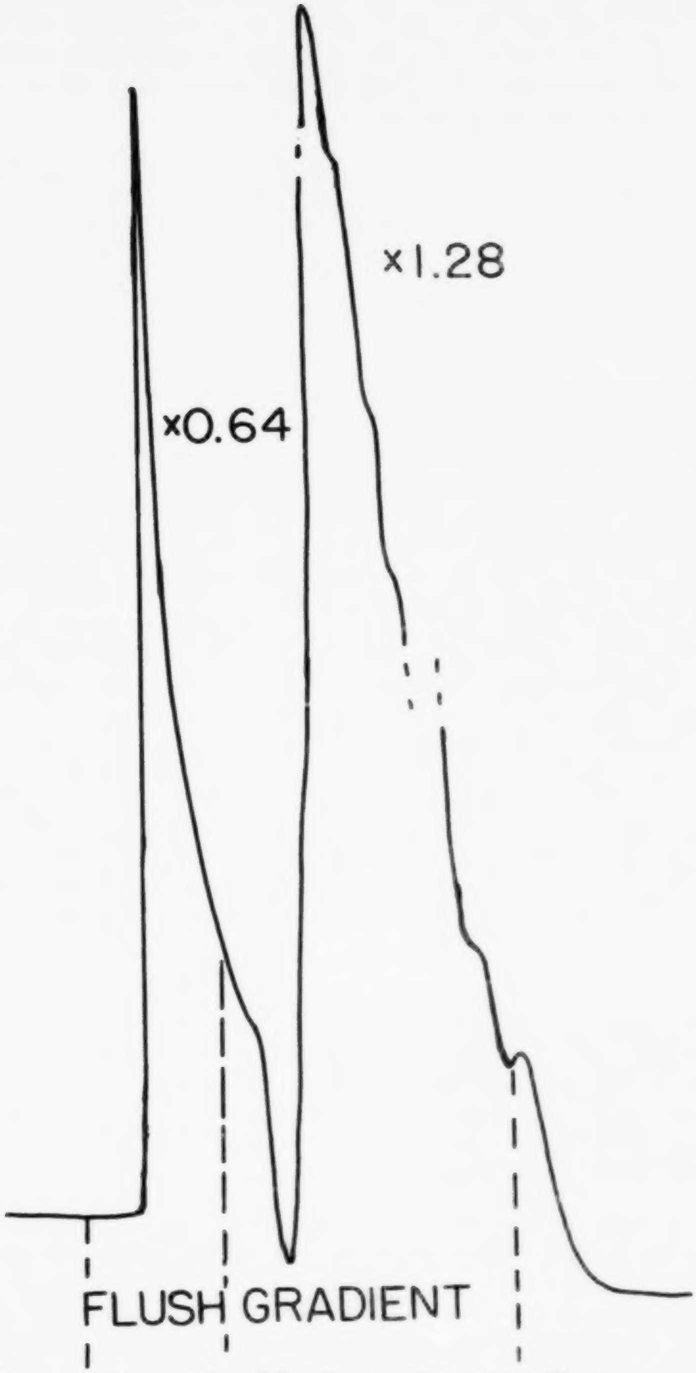


Figure 14. Liquid chromatogram of one liter of Pomona, California secondary effluent; pH 7.8.

We were able to produce an artifical "flush peak" from distilled water by adding benzoic acid, but only if we also added a salt like sodium sulfate or nitrate in a concentration 0.001 M or so. The UV spectrum of this flush peak was that of the benzoate ion, not benzoic acid.

Clearly, the "flush peak" represents material that is weakly absorbed by the Bondapak-C_{18} and is released by passing pure water. The role of electrolytes in forcing the retention of benzoic acid, which is subsequently released by flushing with water, is not obvious and may involve the absorption of sodium benzoate ion pairs. If we flush with a salt solution instead of pure water, no peak appears.

When the methanol gradient is started the UV absorbance first drops, then rises, at first rather slowly, then rapidly. The color of the effluent is at first very pale, then, after the UV absorbance has been rising for 2 minutes, it becomes deep brown. Most of the brown material in the wastewater is concentrated in this peak, which we label "G1". (The G stands for "gradient".) After the UV absorbance reaches its maximum and begins to fall, generally 15 minutes from the start of the gradient, the effluent color changes sharply from brown to blue-green. It remains green for 2-3 minutes (10-15 mℓ), then changes to light brown. Sometimes the absorbance rises to give a small "hump" while the green fraction is passing. We collect the green fraction separately. It is toxic and possibly mutagenic. It has appeared in every wastewater sample we have tested, and its visible absorption spectrum shows a sharp peak at 630 nm. Gel permeation chromatography (see section entitled "Secondary Chromatography and Peak Identification") shows it to contain at least two substances, a green component of low molecular weight and a pale brown component of high molecular weight.

Following the green fraction, the UV absorbance falls and eventually drops to zero some 10 minutes (50 mℓ) after the gradient has ended. (Between the time the liquid leaves the pump and the time it reaches the UV detector, 30 mℓ have passed and 6 minutes have elapsed. Most of the absorbed organic material has been stripped from the column, therefore, before the methanol concentration reaches 100%).

The last fraction, G5 of Figure 13, contains the substances that are least polar, and it is here that most toxicity is found. The G5 fraction is a very pale pink, almost colorless. All the other fractions have a brown color (except green G2), showing that the humic material spreads itself all over the gradient. It complicates the chemical analysis, and frustrates attempts to do gas chromatography-mass spectrometry.

The general form of the elution record for wastewaters is a broad wedge-shaped peak on which is superimposed smaller peaks and minor irregularities. The "envelope" of the peak may be very largely due to humic substances, but we recall that the resolving power of the column is not great, and many individual peaks will merge into a continuum. At this point, the width and form of the "humic acid" peak depends on the pH of the sample that is loaded. The lower the pH, the wider and higher the peak. Figure 15 shows the effect. At the lowest pH (3.9) the envelope is broad and carries at least five distinct peaks. The flush peak, meanwhile, is small. As the pH is raised, the peaks in the gradient region disappear one by one, and the envelope is thinned down. At the same time the flush peak grows higher. The obvious interpretation is that weak acids are present that are retained by the column in their undissociated forms (HA) but not in the ionic forms (A^-). The ionic forms come out in the pre-flush stage or are somehow transferred to the flush peak, perhaps through the retention and subsequent release of ion pairs.

The effect seen in Figure 15 makes it necessary to control the pH of the samples before they are loaded on to the column. A high pH is preferred to a low pH, because toxic substances are likely to be non-ionic compounds like chlorinated pesticides and polycyclic aromatics, whose retention is independent of pH, and at high pH there is minimum retention of unwanted humic acids. The pH may not be raised above 8, we repeat, because the bonded silica column packing would be attacked.

Elution of Known Compounds

A number of known compounds were loaded onto the column by the standard procedure, starting with distilled water or tap water "spiked" with the compounds at concentrations of 1-2 ppm. The elution times depended on the size of the column, the flow rate, and the gradient. Times were measured early in this research, and different columns and conditions were used. The sequence of elution was the same in all the columns, however. Table 6 shows the elution sequence, and approximate retention times referred to our standard 1-cm by 50-cm column.

Compounds that eluted early, like phenol and caffeine, may have moved down the column during the water flush, but doubling the flush period reduced the retention time of caffeine by less than one minute. The prescribed 15 minute flush period is, therefore, not critical.

The sensitivity of detection was estimated by introducing known 5-microgram quantities of caffeine. The peak height under standard operating conditions was 0.02 absorbance units at 275 nm. In two liters of water this represents a concentration of 2.5 ppb ($\mu g/\ell$). This is very satisfactory, but we should note that in high performance liquid chromatography we detect

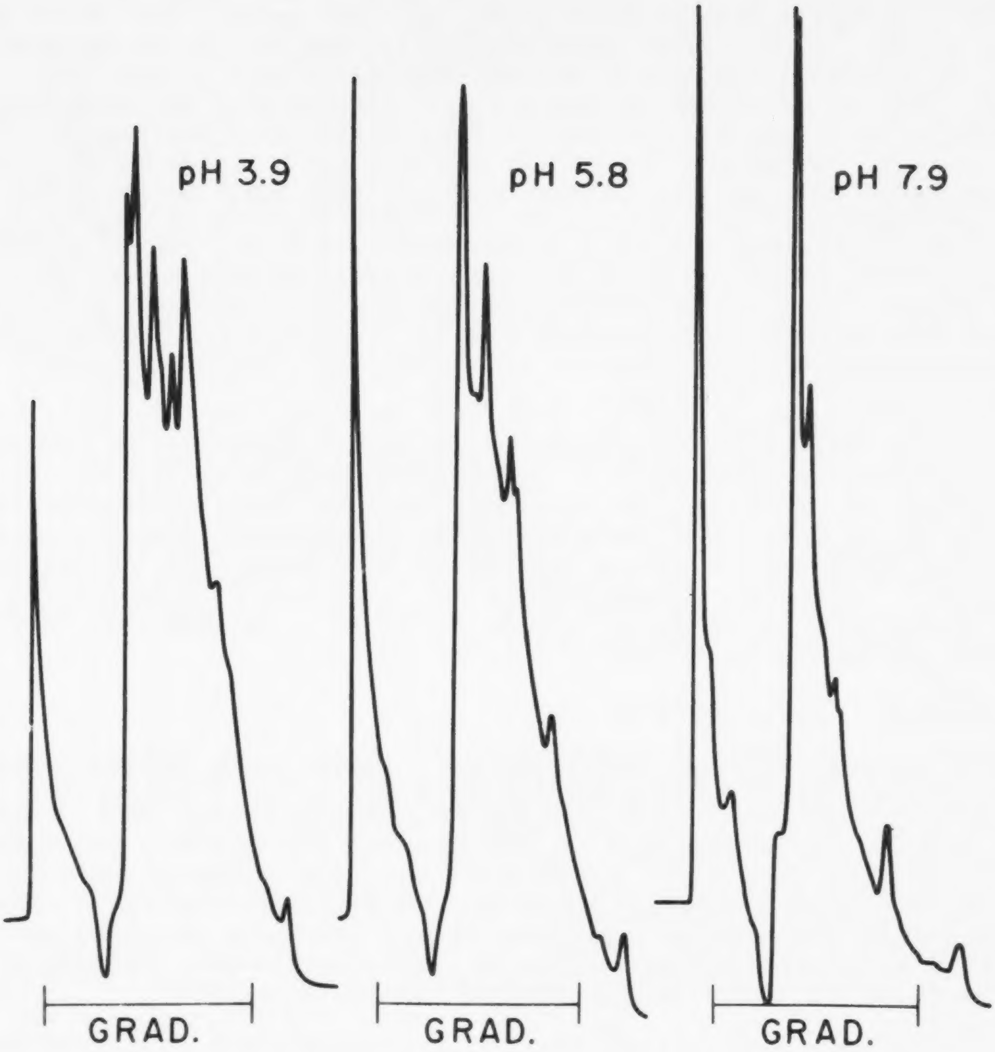


Figure 15. Liquid chromatogram of Denver secondary sewage effluent showing the effect of pH on retention; full-scale absorbance - 0.64 unit.

10 ng of caffeine, because the peaks are so much narrower with short columns and fine packings.

Liquid Chromatography and Toxicity of Treated Wastewaters

In October, 1977, the Denver sewage plant tested, on a small scale, the effects of reverse osmosis and activated carbon on the secondary effluent. We obtained samples of the products and ran chromatograms, with the results shown in Figure 16. Both treatments reduced the organic content considerably. Comparing Figure 16 with Figure 15, for instance, one should note

TABLE 6. ELUTION SEQUENCE ON C-18 COLUMNS

Compound	Minutes from start of gradient
Phenol	19
Caffeine	20
Benzyl alcohol	22
Benzaldehyde	25
Meta-cresol	26
Meta-chlorophenol	28
Ethyl benzoate	29
Diethyl phthalate	30
Atrazine	30
Naphthalene	32
Dibutyl phthalate	34
Diphenyl oxazole	35
Pyrene	36

Note: This is a composite of results from three columns packed with octadecyl silica. The elution times are scaled to our "standard" 1-cm x 50-cm column at 5 mℓ/min with a 30-minute linear gradient.

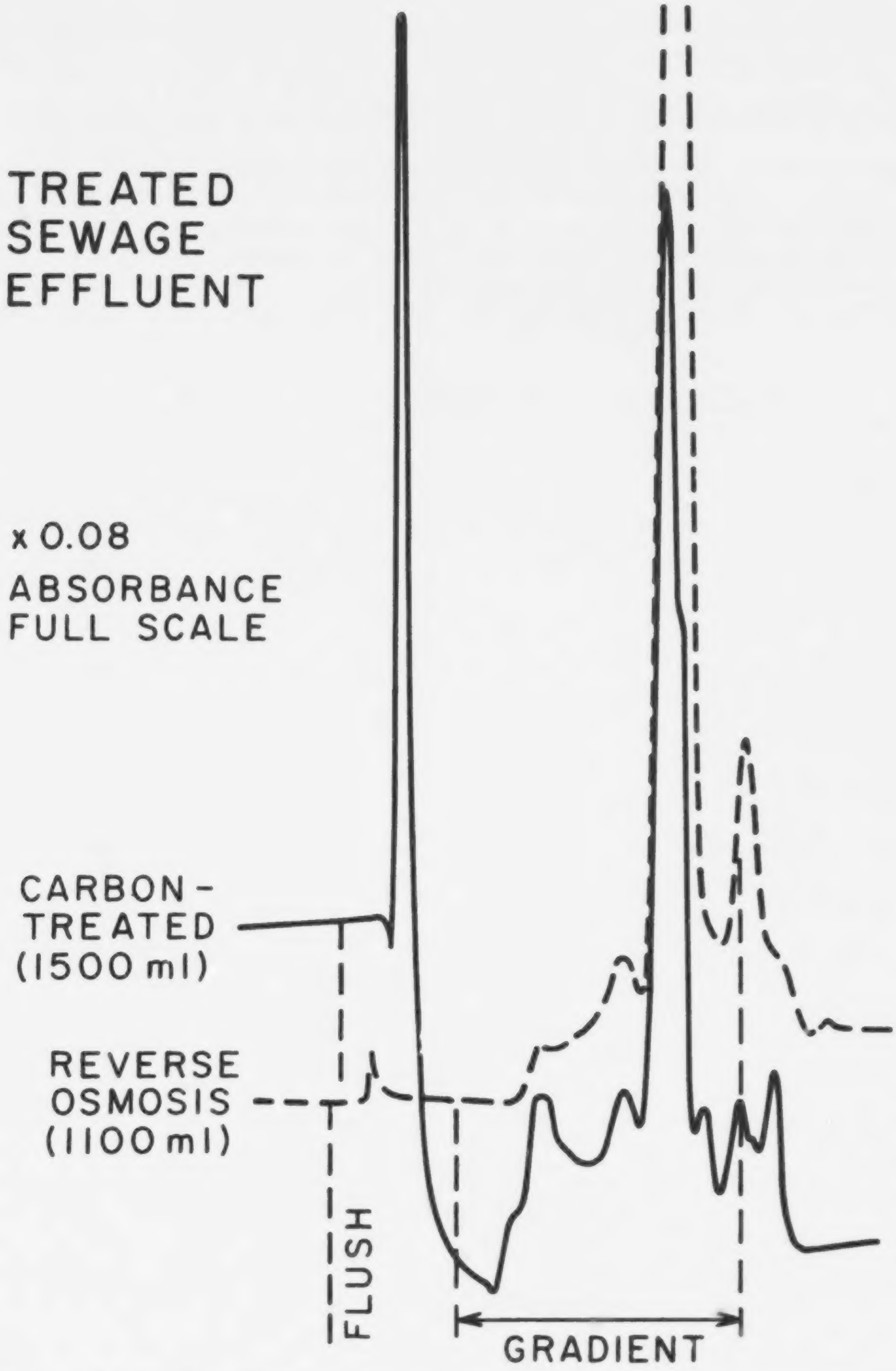


Figure 16. Chromatograms of Denver secondary sewage effluent after the additional treatments shown.

that full-scale deflection was only 0.08 absorbance units in Figure 16, but 0.64 units in Figure 15.

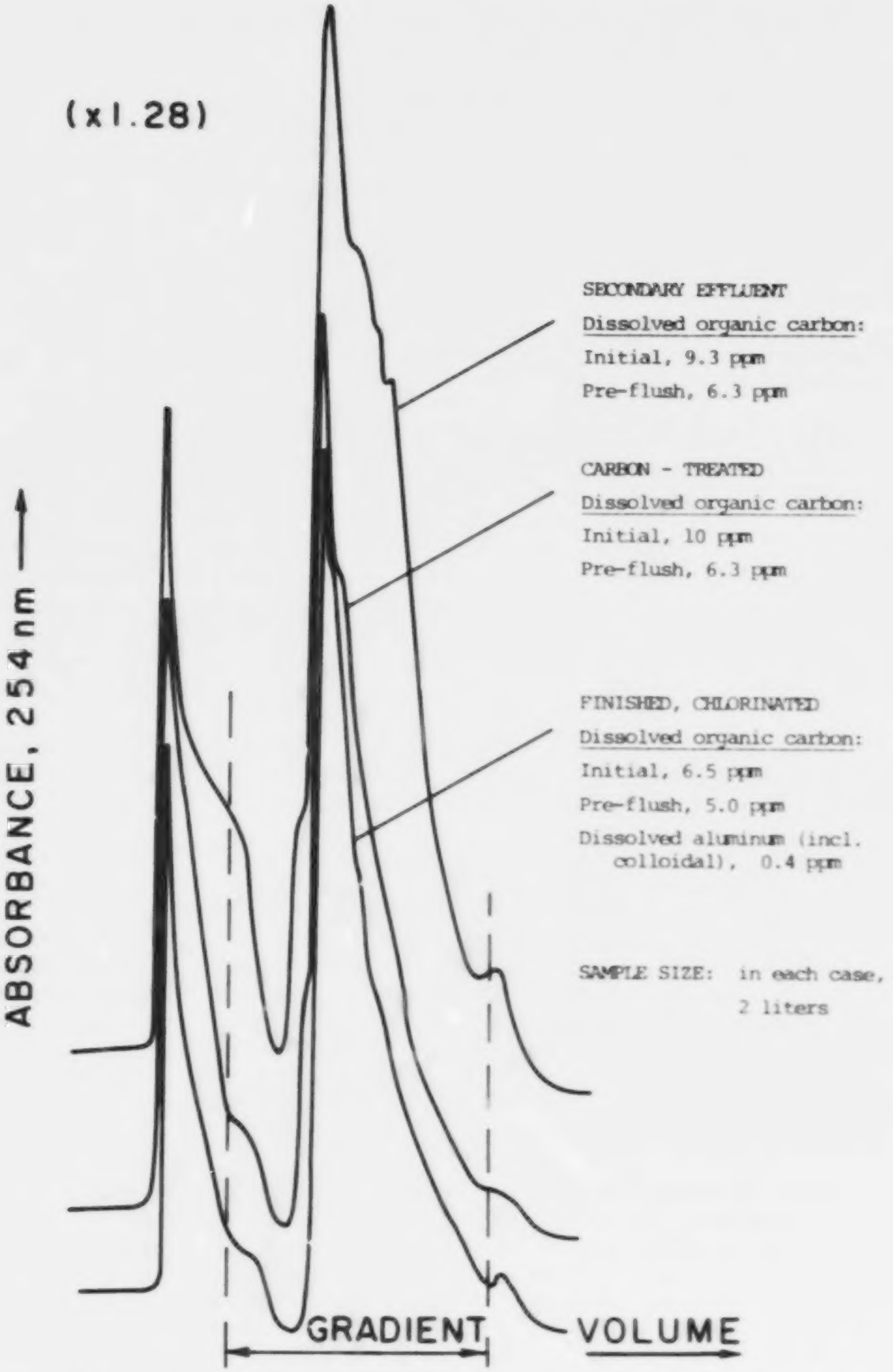
Reverse osmosis suppressed the flush peak and the pre-flush absorbance, indicating that it removed all electrolytes, including inorganic salts and ionized organic acids. The salt content was checked by titration and was below 10^{-4}N. Carbon treatment does not remove inorganic salts and only partially reduces the inorganic acid content (as indicated by the flush peak). Both treatments remove the big humic acid peak, and both let pass some material later in the gradient, corresponding to our G3 fraction. We were not able to make toxicity tests with these fractions because the Denver pilot plant shut down just after we took our samples.

Samples were also obtained, before and after carbon treatment, from the Pomona, California treatment plant. The liquid chromatography curves showed carbon treatment to be effective (see Figure 17). The treatment does not remove the flush peak, though it thins it down, and it does not remove the most polar part of the humic material, but it does remove the less polar material, especially that corresponding to our G4 and G5 fractions. Measurements of total organic carbon showed an overall reduction of about one-third, the pre-flush or polar material being reduced only 20%.

The tests of toxicity showed (1) that most of the toxicity was at the non-polar end of the gradient, and (2) that carbon treatment reduced this toxicity.

To test reverse osmosis we purchased a laboratory water purifier, Milli-RO4, from the Millipore Corporation, Bedford, Massachusetts, and modified it so that a limited volume of feed water could be re-circulated. Eight liters of Denver secondary sewage effluent were treated and divided into 4 liters that had gone through the membrane (the permeate) and 4 liters that had not gone through the membrane (the reject). The chromatograms are shown in Figure 18. The volumes indicated are those of the samples that were loaded onto the chromatographic column; thus, the difference between the permeate and the reject is greater than the chromatograms show.

Reverse osmosis reduced the total organic carbon considerably, and removed electrolytes, suppressing the pre-flush absorbance and the flush peak. Once again, reverse osmosis allowed some material to pass in the middle of the gradient, and there was evidence that non-polar and weakly polar substances were passing. Toxicity tests confirmed that some of the undesirable weakly-polar substances were passing the membrane, but they showed that on the whole, reverse osmosis was effective in reducing the toxicity.



SECONDARY EFFLUENT
Dissolved organic carbon:
Initial, 9.3 ppm
Pre-flush, 6.3 ppm

CARBON - TREATED
Dissolved organic carbon:
Initial, 10 ppm
Pre-flush, 6.3 ppm

FINISHED, CHLORINATED
Dissolved organic carbon:
Initial, 6.5 ppm
Pre-flush, 5.0 ppm
Dissolved aluminum (incl. colloidal), 0.4 ppm

SAMPLE SIZE: in each case, 2 liters

Figure 17. Chromatograms of three samples of Pomona, California wastewater, collected in May, 1978.

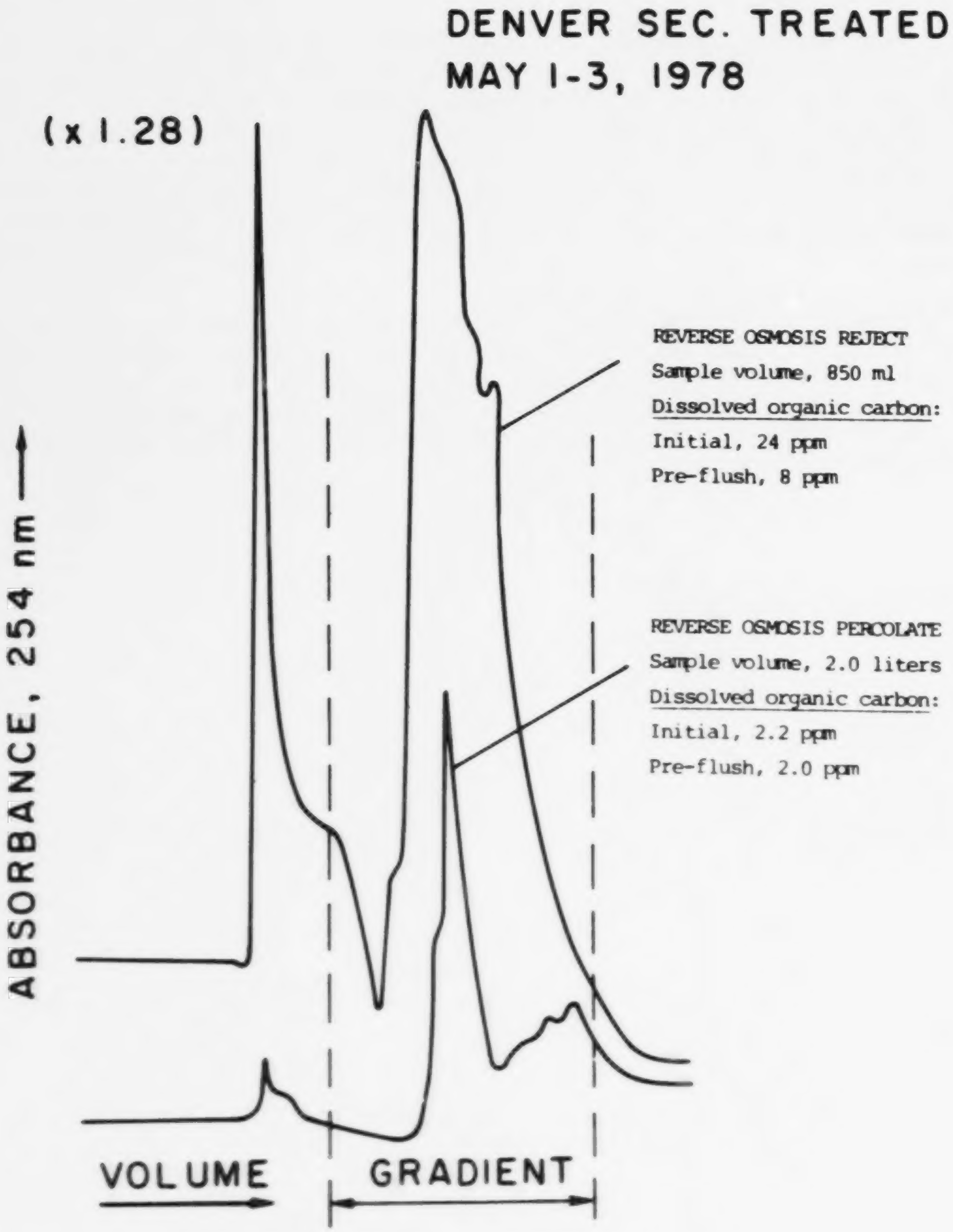


Figure 18. Denver secondary effluent, treated by reverse osmosis in the laboratory.

Liquid Chromatography of Tap Waters and Environmental Samples: Solvent Blanks

Some miscellaneous chromatograms are included here. Since the carbon content of these waters is much less than that of wastewaters, attention to solvent blanks is necessary. Figure 19 shows the blanks with freshly redistilled water and two commercial brands of liquid chromatography grade methanol. The water was purified as described above. The XAD-2 resin filter used to clean the house distilled water must be changed and refluxed with methanol in the Soxhlet extractor mode every month or so.

Figure 20 shows a chromatogram of the pristine spring water that is described above in the section on volatile compounds. Ionized organic compounds are present, as well as the ubiquitous humic acids. The small peak at the end of the gradient is due to the solvent blank. Note that full-scale deflection is only 0.08 absorbance units.

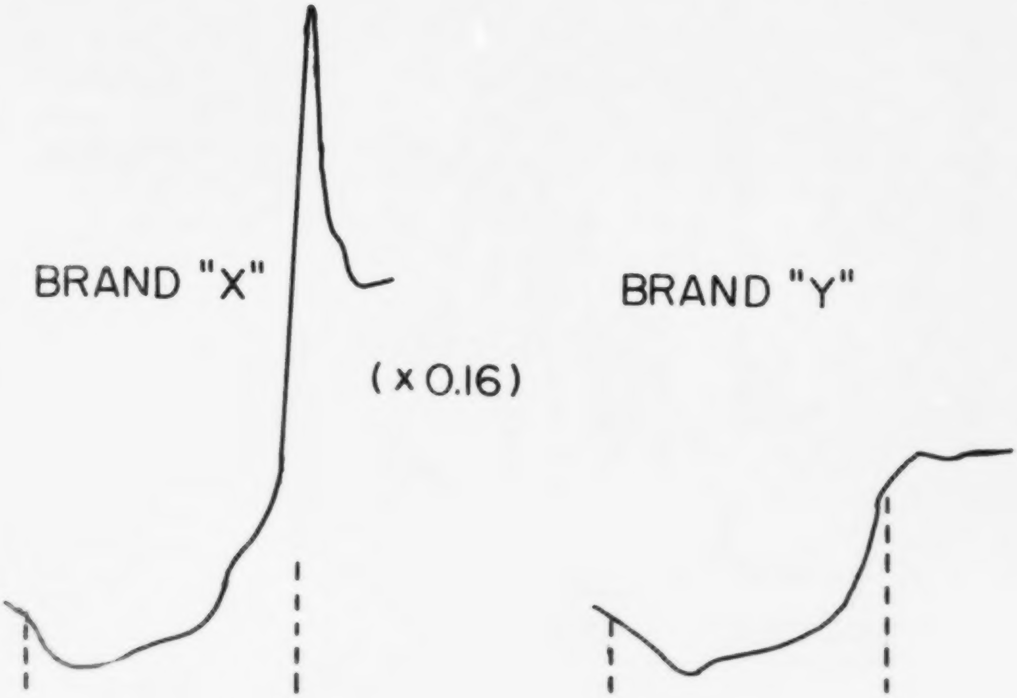


Figure 19. Blanks with redistilled water and liquid chromatography-grade methanol.

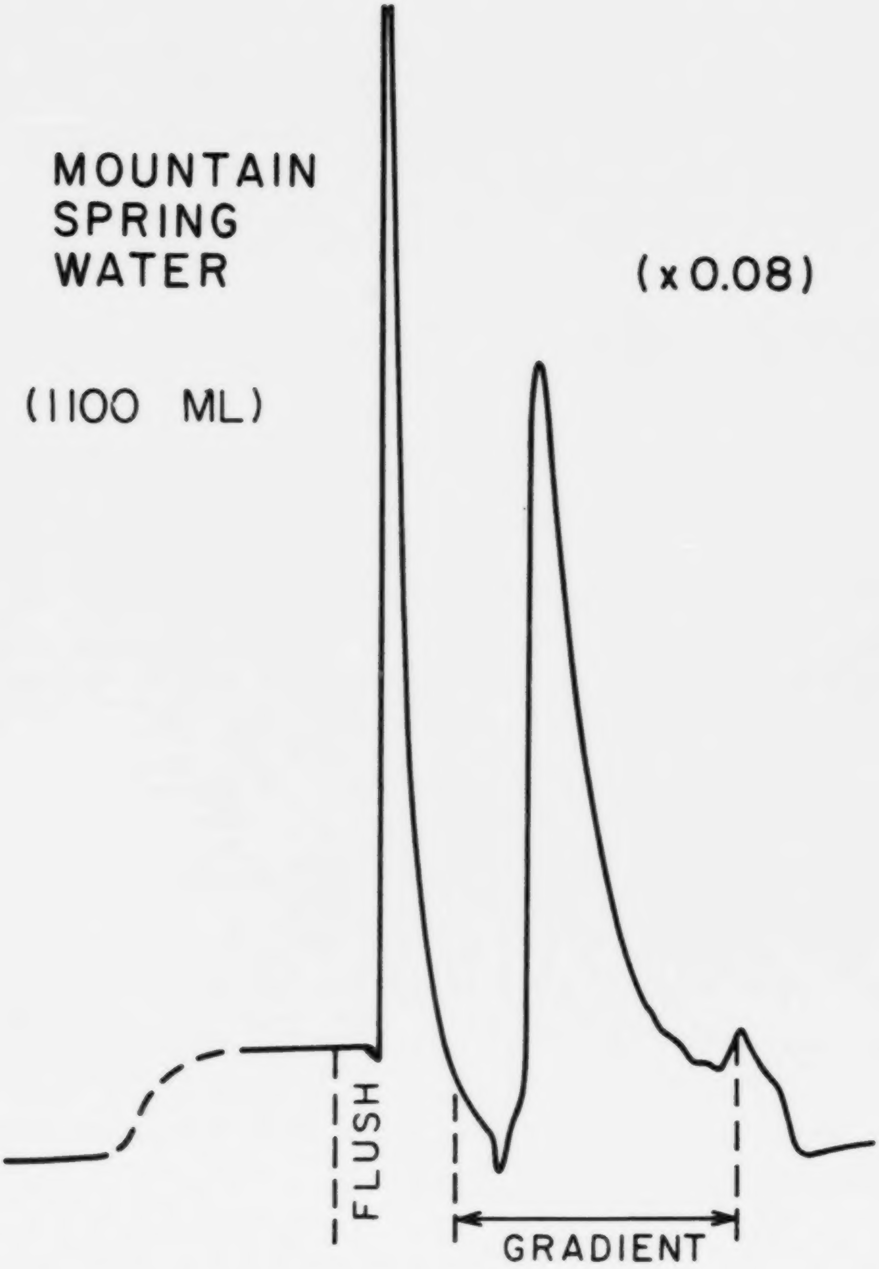


Figure 20. Chromatogram of a "pristine" mountain spring water.

Chromatograms of Denver tap water are shown in Figure 21. The effect of pH, previously discussed, is clearly seen, and it is evident that this water contains considerable humic material. The sharp, high peak beyond the end of the gradient was spurious. It was caused by a surface-active agent of formula $(C_3H_7)_3C \cdot C_6H_4(O\ CH_2CH_2)_n \cdot OH$, which was coming out of the Millipore HAWP filters we were using at that time. (Identification was made by mass spectrometry and ultra-violet spectrometry). We changed to polycarbonate membrane filters, and then, when we confirmed that these filters could absorb dissolved naphthalene from water, we avoided membrane filters altogether and used only glass-fiber filters.

Figure 22 shows chromatograms of Boulder tap water; Figure 23 shows water of the South Platte River in Denver, above the sewage plant discharge, and water from a shallow well in Boulder, 0.5 kilometers down from the old, now abandoned plant of Arapahoe Chemical Company. These two chromatograms were early ones obtained with the microparticulate, analytical C-18 column. They show the resolution that such columns will give, and Figure 23b shows the complexity of this unusual water.

Secondary Chromatography and Peak Identification

The resolving power of the large Bondapak column is poor. The absorbent, Bondapak C_{18}-Porasil B, was chosen because it would absorb and desorb the constituents of filtered sewage over and over again with no irreversible sorption; moreover, it is easy to pack and gives little back pressure. With this column there is no hope of isolating individual compounds, save in exceptional cases like the surfactant from the filters that appeared as a spurious peak in Figure 21. Each fraction, G1 through G5, contains many compounds of moderate molecular weight, as well as the ever-present humic substances. To separate individual compounds and to have any hope of identifying them, it is essential to use columns of high resolving power, and preferably columns and solvents that have different kinds of selectivities. The idea is to collect fractions from the first column, concentrate them by evaporation in the manner described, and then inject them into the high-resolution column.

So far, three kinds of absorbents have shown promise as packings for secondary high-resolution columns. One is a strong base anion-exchange resin (Aminex A-25, Bio-Rad Laboratories); using buffered alcohol-water mixtures, we have achieved good chromatography of the flush peak, which contains very polar compounds, and have also been able to separate the less polar fractions (see Figure 24). Another class of packings is the porous polymer gels. Porous polymers discriminate between solutes in two ways: size exclusion and sorption-partition. Several kinds of polymers are available, with different kinds of polymer matrix (polystyrene, polyacrylate, etc.) and different

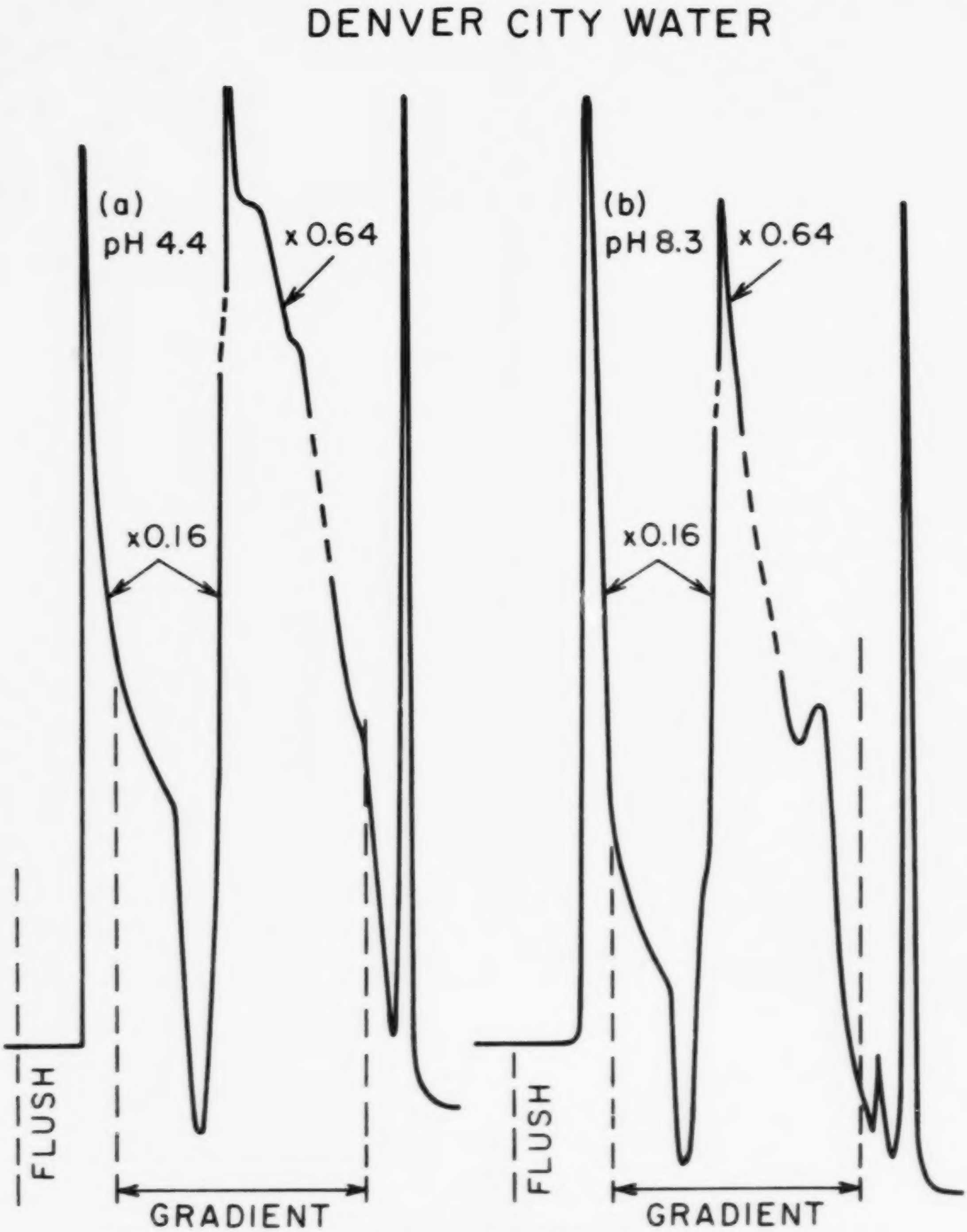


Figure 21. Chromatograms of Denver tap water showing effect of pH.

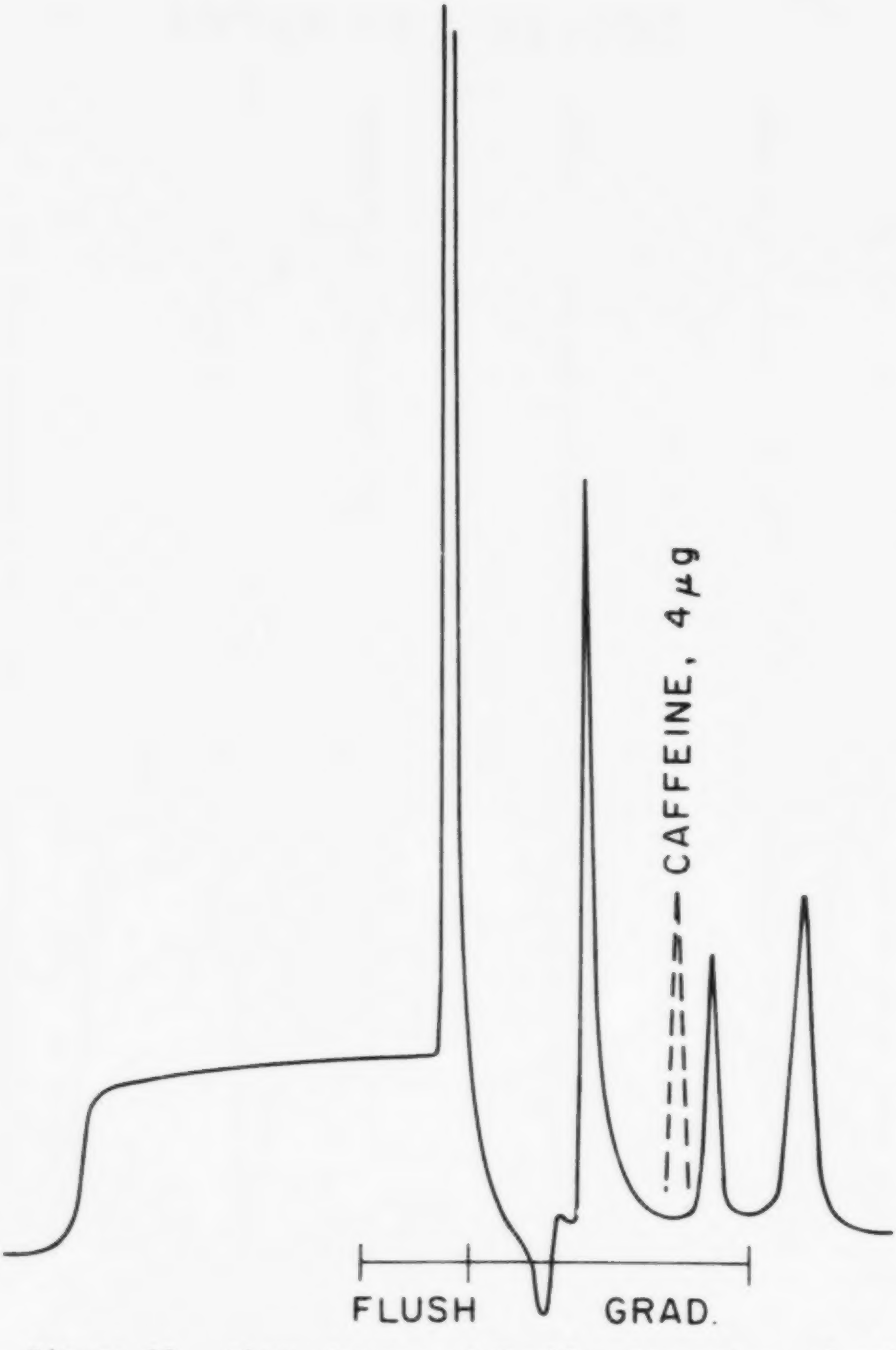


Figure 22. Chromatogram of Boulder tap water, showing effect of the addition of caffeine.

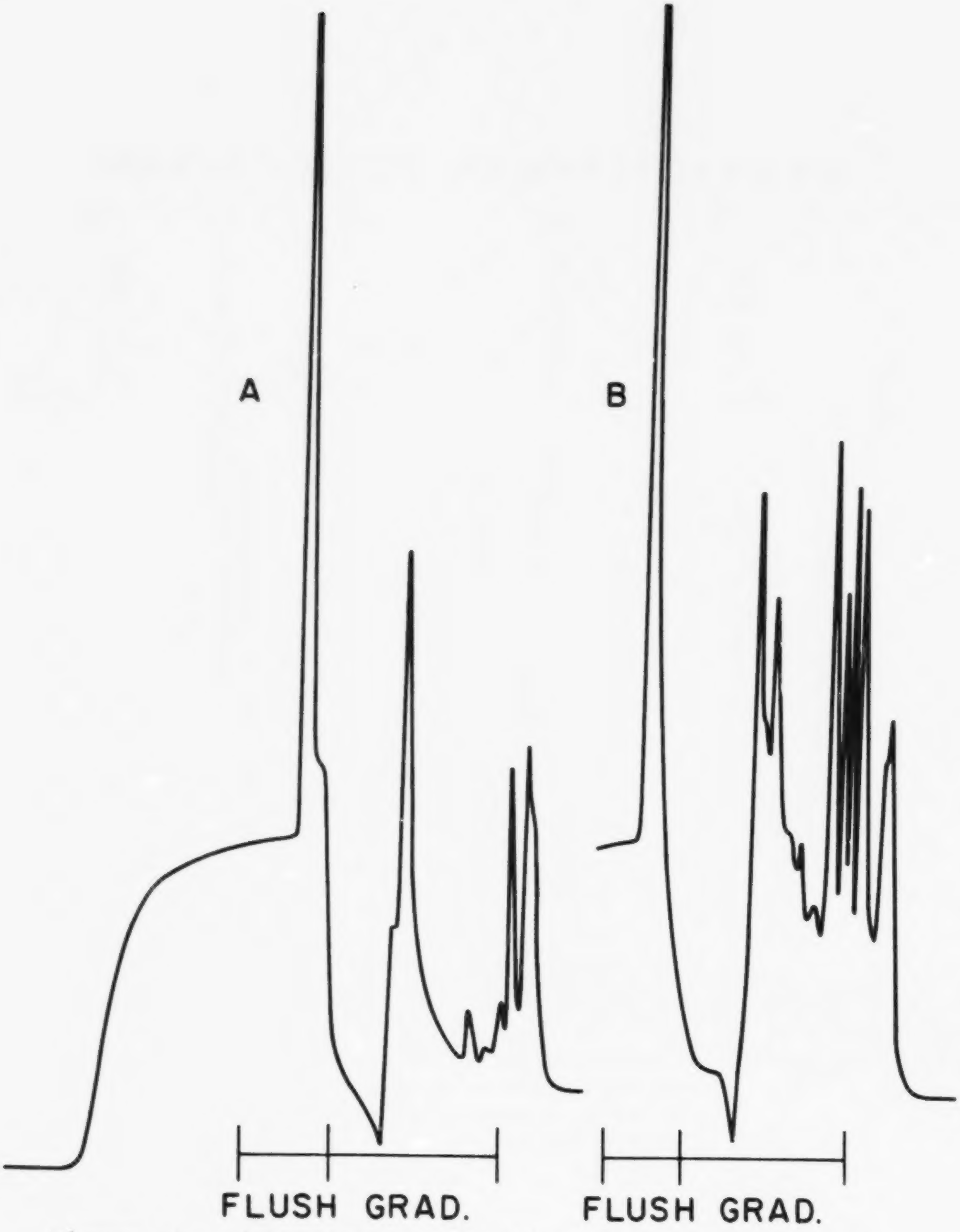


Figure 23. Chromatograms of (A) of South Platte River above sewage plant, and (B) of a highly contaminated shallow well near Boulder.

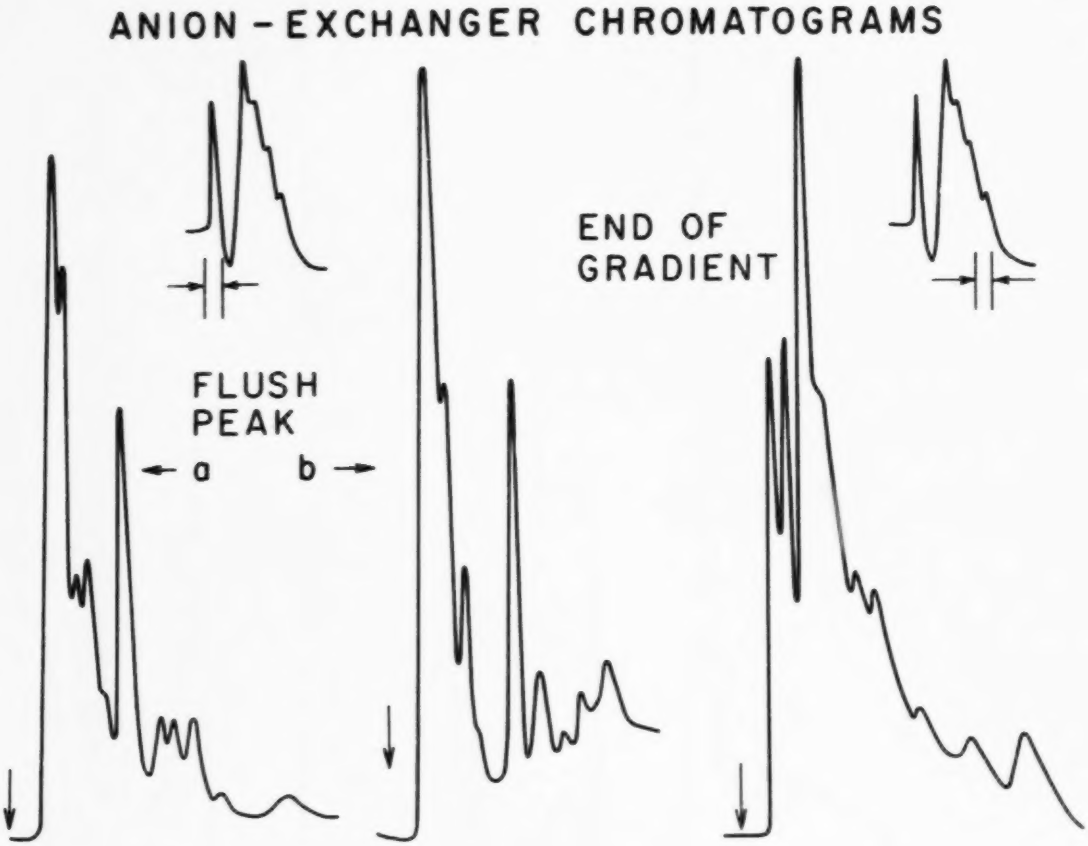


Figure 24. Chromatograms of flush peaks and a weakly polar fraction on anion-exchange resin; eluent: acetate buffer, pH 5.5 in 25% ethyl alcohol.

functional groups. Materials of small particle size, specially designed for liquid chromatography, have become available from Japanese manufacturers in the past year or two. The third class is the microparticulate bonded packings that are so widely used in liquid chromatography today. The most popular of these, and the only one we have worked with to date, is the microparticulate C_{18}-bonded silica. Chemically this is the same material as we used in our main column, but the particle size is much less, the available surface area is much greater, and there are other minor differences. The loading capacity is small, but the resolution is excellent.

Figure 25 shows chromatograms obtained on porous polymers. The solvent in each case was 55% (v/v) acetonitrile, 0.02 molar in tetrabutylammonium hydroxide. Curve A shows the green G2 fraction on Hitachi 3010 polystyrene gel; curves B and C are for G3 and G4, respectively, on Toyo Soda TSK-LS 110 polystyrene gel. The peaks appearing earliest are those corresponding to the highest molecular weight. Not shown are some preliminary data from the prepacked Toyo Soda column of a hydrophilic gel, G-2000-SW, which establishes the molecular weight range in fractions G1 and G3 to be 20,000 and below. The resolution of the green fraction, G2, into two components has been mentioned; the first, of high molecular weight, is a light brown color; the second, which is blue-green, probably has a molecular weight below 1,000. Fraction G3 is clearly very complex, while fraction G4 is neatly divided into a portion of high molecular weight that comes out first, followed by a series of compounds of relatively low molecular weight (below 1,000) that is partially resolved.

We are on the threshhold of solving one problem that has perplexed us, the problem of removing the high-molecular-weight humic substances in order to facilitate chemical analysis of the simpler compounds. We see three ways to approach this problem:

1. Absorb the humic acids on an anion-exchange resin column. Inspection of the resin shows that they are retained very strongly as a narrow, dark brown band at the entrance to the column.
2. Use size-exclusion porous polymer gels, as just described.
3. Filter the solutions through membrane ultrafilters like the "Diaflo" (Amicon B.V., The Netherlands) ultrafilters used to separate proteins. We have seen that these filters can fractionate our G1 samples.

The least polar fraction from the main column, fraction G5, contains very little humic substances. We therefore injected it directly into a microparticulate bonded C_{18}-silica column, MicroBondapak-C_{18} (Waters Associates). With this column we used the same solvent system as in the main column, namely methanol

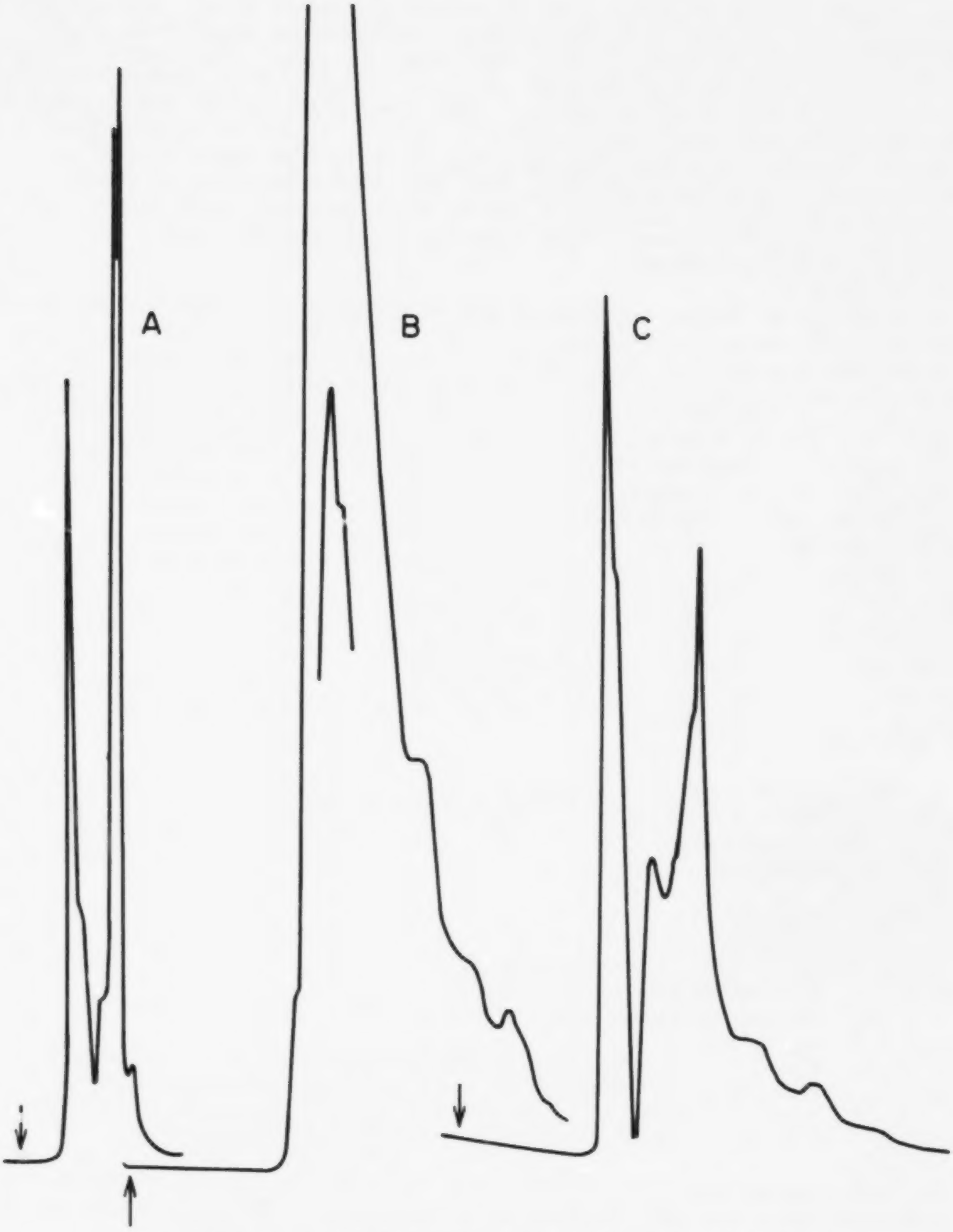


Figure 25. Chromatograms of: (A) G2, (B) G3, (C) G4 on porous polymers.

and water. The procedure was the following:

The G5 fractions from 10-20 liters of secondary sewage effluent were evaporated nearly to dryness, then methanol was added to give 3-4 mℓ of a concentrated solution that was about 70% in methanol. Portions of this concentrate were injected into a stream of 60% methanol by means of a valve and sample loop; they passed into a MicroBondapak-C_{18} column, at the outlet of which was an ultraviolet detector, and in some experiments a fluorescence detector also, connected downstream from the UV detector. The flow rate was 0.5 mℓ/min. The 60% methanol was passed for a while, generally 20 minutes, then the methanol concentration was raised from 60% to 100% over 30 minutes according to a linear program. The UV absorbance and fluorescence were recorded.

Curves like those in Figure 26 were obtained. The heights of the peaks relative to one another varied somewhat from one collection of sewage to another, but the positions (retention times) were the same, and the very sharp peak near the end of the gradient was always present.

As a first step to identification, the peaks were collected and their ultraviolet absorption spectra and fluorescence spectra were measured. Then the solutions from each peak of interest were evaporated in weighed vessels at room temperature, and the weights of the residues were found. The residues were dissolved in appropriate solvents (carbon disulfide, carbon tetrachloride) and the infrared spectra run. Solutions of the residues in methanol were examined by mass spectrometry, using heated-probe injection and also gas chromatography-mass spectrometry.

As another aid to identification, once we had decided on a solvent program (20 minutes isocratic flow with 60% methanol, 30 minutes linear program to 100%), we injected a number of known compounds and noted their retention times.

The high, sharp ultraviolet peak of Figure 26 was studied in detail. Its retention time is close to that of dibutyl phthalate. It is obvious from visual inspection that this peak is complex. Watching the drops of solution as they emerge from the detector, one sees a pink color appearing just before the sharp rise in ultraviolet absorbance. The solution collected over the UV peak is pink, and when it is evaporated, a bright red solid speck appears before the solvent is all gone. The bulk of the residue is a colorless liquid. The absorption spectrum of the solution has a peak at 547 nm, corresponding to the red color, and a much stronger peak in the ultraviolet at 262 nm.

Mass spectrometry showed the presence of three compounds.

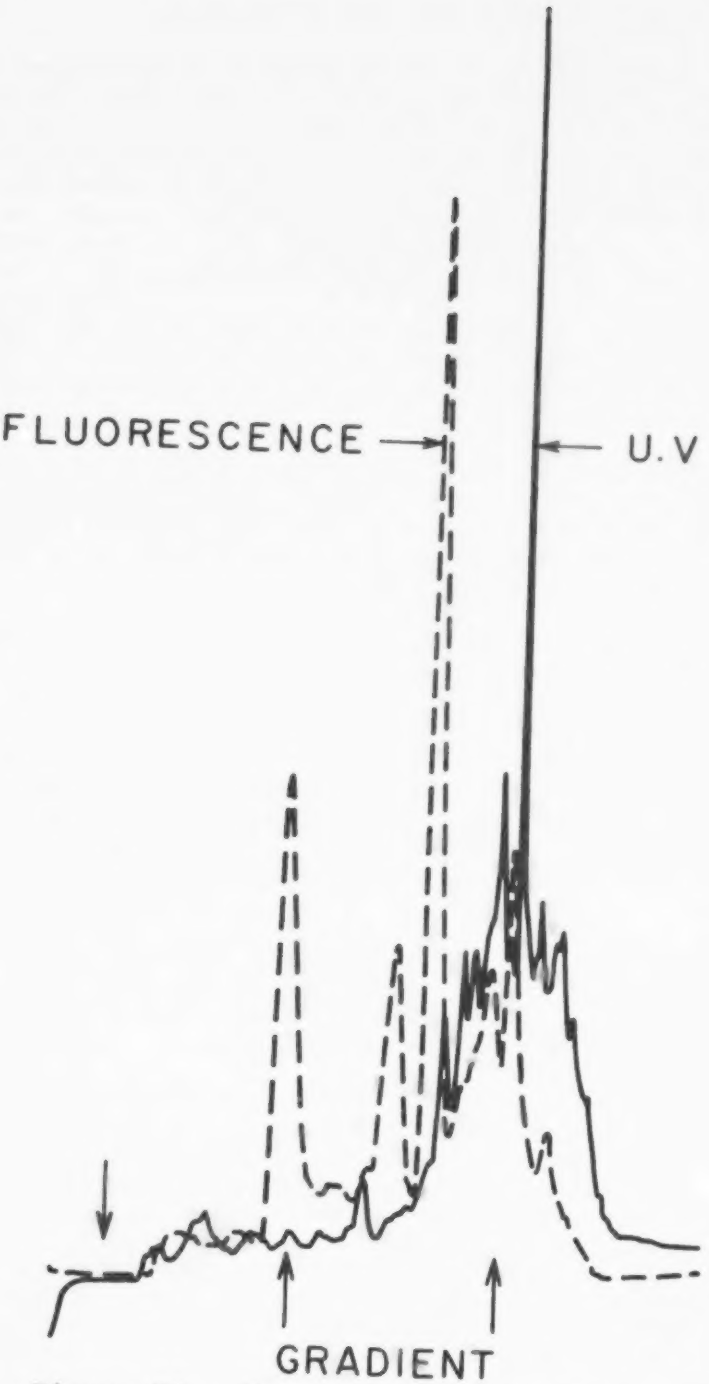


Figure 26. Chromatogram of G5 on MicroBondapak-C_{18}.

The most prominent and the least volatile is tri(2-butoxyethyl) phosphate, a common plasticizer. This compound does not absorb in the ultraviolet, and it was a coincidence that it came out of the column along with a strong ultraviolet peak. The other compounds were phthalate esters, one being dioctyl phthalate. Yet phthalate esters have ultraviolet absorbance maxima near 274 nm, not 262 nm. The observed 262-nanometer peak must be due to another substance. A search of Grasselli's Atlas of Spectral Data suggests some possibilities, but we have not positively identified this compound.

Counting the red substance, which we have also not identified, there are at least five compounds in this one sharp ultraviolet peak. The peak that follows the high peak is also complex, to judge from the mass spectrum; the mass spectrum corresponds to a nonylphenol ether surfactant, yet the absorbance maximum is at 275 nm, which suggests a phthalate ester.

The infrared spectra were of little help. Both fractions showed weak bands due to oxygen-carbon vibrations. The weights of the residues were less than a milligram, and corresponded to concentrations in the original filtered wastewater of 100 and 150 ppb (micrograms per liter) for the two peaks collected, assuming no loss in processing. One can see the magnitude of the analytical problem, and the need for processing large volumes of wastewater.

SUMMARY

We are devising methods for the chemical analysis of the very complex mixtures of organic compounds found at trace levels in natural waters, treated waters, and wastewaters. One part of our problem has been essentially solved, that is the identification and measurement of volatile substances that can be swept out of water by warming and bubbling nitrogen. These substances include chloroform, other chlorinated hydrocarbons, benzene and toluene, all of them substances that are toxic to some extent. They are trapped on a special absorbent (Tenax) and transferred to a gas chromatograph, where they are analyzed and identified by mass spectrometry. Concentrations of one part per billion and less can be measured. Applying our technique to wastewater and drinking water, we see what substances are present and how they are affected by treatments such as chlorination, ozonation, and filtration through activated carbon. Carbon treatment is being introduced on a very large scale for removing organic compounds from drinking water and wastewater; our findings suggest that it removes toxic substances but is not especially effective in removing traces of volatiles.

The analysis of organic compounds in water that are not volatile is a much more difficult problem. Nobody has found a satisfactory solution, but we have made a start. We have been able to "strain out" or selectively absorb a part of the organic material, using an absorbent called Bondapak-C_{18}, followed by stripping the absorbed substances off the Bondapak by a series of mixtures of water and methanol. We obtained several fractions, graded them according to "polarity", or compatibility with water, then concentrated them by evaporation so that the substances originally present in say 10 liters of wastewater, are recovered as a series of six fractions, each of volume 4 mℓ. To guide the fractionation process we record the absorption of ultraviolet light. The record on the chart paper lets us see what is going on, and lets us compare the results of various wastewater treatments.

The concentrated fractions are submitted for cellular toxicity tests. Lately, we have also been testing samples for mutagenic activity. Our Bondapak absorbent retains only one-third of the total organic carbon in wastewater, but the non-retained material does not seem to be toxic. The toxicity is concentrated in the less polar fractions. It is here that we would expect to find pesticides and products of industrial contamination. As to mutagenic effects, we have tested four sets of samples and the data are not consistent. In some tests the polar fractions were found to be strongly mutagenic, particularly the green G2 fraction, while the less polar fractions were not. In another test, G1 was mutagenic but G2 had little effect. Clearly, the composition of a municipal sewage effluent varies from day to day, and perhaps we should not expect consistency. Mutagenesis may be due to a minor component appearing on the border between G1 and G2, a component whose concentration varies greatly from day to day. Mutagenesis is a serious matter, however, and more tests will be made. The cellular toxicity of the less polar fractions has been confirmed many times. We have used the cellular toxicity tests to judge the effectiveness of carbon treatment and reverse osmosis.

The next step is a more detailed chemical analysis of the various fractions and the identification of specific chemical compounds that are responsible for the toxicity. We are attacking this problem with advanced techniques of liquid chromatography. To make progress we need to refine our separation technique, and to collect toxic fractions from much larger volumes of wastewater.

SECTION 4

TOXICITY TESTS: CELLULAR METABOLIC STUDIES

INTRODUCTION

To determine the total health effects of a substance in the environment is a formidable task. Because of the complexity of mammalian cellular metabolism and the large variety of thousands of interacting chemical reactions in the body, it is impractical to test a suspected toxin against every known metabolic step or sequence of steps. Even if this could be done, the ability of the cell to compensate, within limits, for many types of stress would make interpretation and extrapolation extremely difficult. The situation is simplified if the compound being evaluated has a high specificity for a single enzyme system. For example, organo phosphates (Parathion) specifically inhibit acetylcholinesterases and quickly disrupt the body's neuro-muscular system. However, apart from acute toxicity of this type, which is relatively easy to detect, we are more concerned about the effects of chronic exposure to much less acutely toxic substances of low concentrations. Therefore, a variety of compounds, including aromatics, paraffins, and their chlorinated derivatives, were studied, as well as fractions of unknown compositions derived from treated sewage and purified water. We also wished to measure the relative effectiveness of water purification steps such as charcoal treatment and reverse osmosis with a view to developing on-line biological testing as described in a new grant proposal.

The main difficulties of of obtaining meaninful data can be categorized as follows:

1) Concentrations of test substances are low (from 1.0 to less than 0.1 ppm).
2) Exposure time for rapidly metabolizing cells *in vitro* is limited to 30-300 minutes to avoid the effects of normal deterioration of cellular metabolism.
3) Of all the possible pathways, which are the ones to be selected as the most valid as test models?
4) If cellular toxicity is detected *in vitro*, how does one extrapolate this data to evaluate gross human toxicity?

During the period of this grant considerable progress has been made in dealing with these difficulties, although the problem has not been solved in its entirety. Nevertheless, an understanding of the short-term effects of chemicals on metabolic reactions has led to a reasonably accurate prediction of their effects in man (12) and is in general agreement with available data on gross toxicity.

CHOICE OF A METABOLIC PATHWAY

In attempting to deal with the problems of low concentration and relatively short periods of exposure, it is essential to use cells that are extremely sensitive to a very small concentration changes in their immediate environment, on the order of 10^{-6} - 10^{-5}M, and which react explosively and uncontrollably once they are triggered. Two types of cells, platelets and leucocytes, which normally fulfill these requirements in the body, were studied.

Normally, when a blood vessel is cut or damaged the platelets aggregate to each other to form a plug which stops bleeding and acts as a catalytic surface for further coagulation. In the course of aggregation the platelet becomes degranulated, releasing a number of substances, including ADP, serotonin and a variety of proteins. Platelet function is linked closely to adenylate metabolism (13) and is regulated by membrane function. Consequently, lipid soluble chemicals which change membrane function can affect platelet metabolism and behavior. This concept is important not only from the viewpoint of hemostasis, but is also related to the role of platelets in forming unwanted plugs in the lumen of arterial vessels, thus contributing to thrombotic coronary disease, a major cause of death.

Another type of cell, which also functions explosively to stimulus, is the white cell in the blood which forms one of the defenses against infection and malignancy. These cells can ingest or phagocytose bacteria and kill them with hydrogen peroxide generated metabolic action. Thus, substances which impair these functions or cause these cells to inappropriately attack the tissues of the host are of concern. The white cells, like the platelets, generate and use large quantities of high-energy-transducing purines (ATP, etc) and have efficient pathways for salvaging ATP from degradation products such as hypoxanthine (HYPX).

The third difficulty of choosing a relevant pathway to study is not as easily solved and all choices of metabolic pathways are open to some sort of criticism. However, there is one sequence which is of central importance to both the energizing and kinetic control of all metabolic steps. This system is the adenylate control system which has come to be recognized as being of prime importance in energy transduction, storage and control of metabolic rates and directionality (14). The importance of the fast-acting adenylate control system cannot be exaggerated for a large number of chemical reaction sequences which typify living organisms. The adenide nucleotides interact with all sequences in a complex living cell and uncontrolled changes in the relative concentrations of ATP, ADP and AMP would adversely affect the rates of all metabolic reactions and thus be highly disruptive. Therefore, we have developed methods to assay adenylate pools when platelets and white cells are brought into contact with a variety of chemicals.

The basis of adenylate control resides in the fact that these purine nucleotides combine with enzymes and alter the rate and direction of important control enzymes. For example, under stress the AMP/ADP ratio is increased and stimulates the activity of phosphofructokinase (PFK), thus accelerating glycolysis and stimulating ATP production. Isocitrate dehydrogenase is similarly stimulated by AMP, resulting in an accelerated Krebs cycle, with the ultimate regeneration of more ATP. These reactions are appropriate for survival since a significant fall in the concentration of ATP leads to increased AMP by the pathways ATP, ADP, AMP; the AMP mediates correction of the ATP level by stimulating glycolysis and phosphorylation. Similarly, when the demands on the cell are reduced, PFK is inhibited and reserves are conserved. Reactions of this type are normally taking place every instant with the result that ATP levels are maintained at optimal levels.

Another important aspect of this system is that evolutionary design has favored a high equilibrium constant over a large yield of ATP. A high equilibrium constant allows the cell to advantageously use very small amounts of food while paying the price of a reduced energy yield. A higher yield would require a plentiful supply of fuel at all times, and this would reduce the potential for survival. However, since the efficiency of ATP production is relatively low, stressors and toxins have the effect of further reducing the ATP available for useful work and thus seriously reducing the cells' ability to carry out their functions. This is most readily seen in platelets which are unable to make ATP 'de novo' from its building blocks but must rely on scavenging and recycling the purine ring compounds such as adenine and hypoxanthine from its surrounding.

It is remarkable that the role of the adenine nucleotide pool is unique in being involved in virtually every metabolic sequence in the cell. The role of the adenylates is not specific to any single pathway but, more than any other compounds, they couple and correlate all the metabolic activities of the cell, giving rise to biological homeostasis and function. For these reasons, the purine nucleotide system was chosen for our study of health effects of substances in drinking water. An outline of the metabolic pathways utilized in this project is shown in Figure 27. The effect of stress on this pathway is to reduce ATP and increase AMP and hypoxanthine (Figure 28).

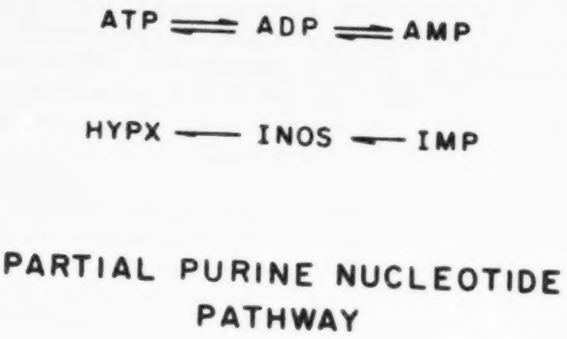

ATP
ADP
AMP
HYPX
INOS
IMP
PARTIAL PURINE NUCLEOTIDE PATHWAY

Figure 27. Partial purine pathway.

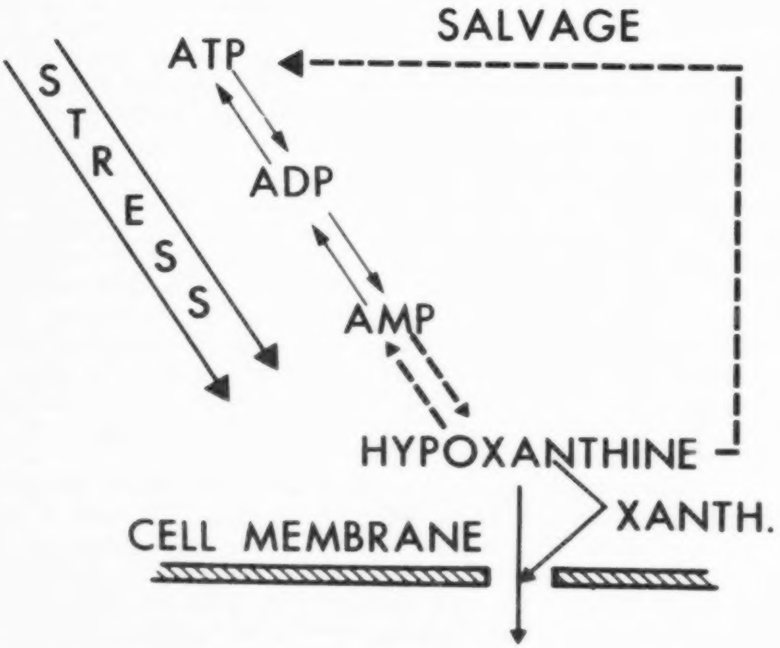

SALVAGE
ATP
STRESS
ADP
AMP
HYPOXANTHINE
XANTH.
CELL MEMBRANE

Figure 28. Effect of stress on ATP metabolism.

METHODOLOGY

Because of the nature of the experimental conditions in which radioactive adenine was used as a precursor, the only radioactive compounds formed in significant quantities were ATP, ADP and AMP, with occasional formation of inosine monophosphate (IMP and HYPX). Dose response curves were used to express the results of known toxic compounds on adenylate metabolism as well as the effects of sewage, tap water and water effluents during treatment. In outline, the platelets were first obtained as a suspension by the standard method of differential centrifugation of anti-coagulated blood. Care was taken to prevent stressing the platelets during blood drawing or subsequently in the laboratory. Thus, only a 'clean' venipuncture from healthy blood donors was used and high speed centrifugation and washing avoided. Suspensions of white cells were prepared and treated similarly.

In a typical experiment, 5μℓ of U-14-C adenine (60,000 cpm) was added to a 0.5 mℓ suspension of platelets (2-5x10^5/cu mm) or white cells (10,000/cu mm). Ten microliters of the test fractions to be evaluated were added. After incubation at 37°C for 30 minutes the cells were cooled in ice and centrifuged at 3000 rpm for 3 minutes. The supernatant was removed and 0.2 mℓ of cold 13% perchloric acid (PCA) was added to the pellet. After mixing and centrifuging, the PCA supernatant was analyzed by thin layer chromatography (TLC) and high performance liquid chromatographic systems (HPLC) described below. The TLC system allows one to measure the kinetic synthetic ability of the cells to convert U-14-C adenine to U-14-C purine nucleotides. The HPLC system measures the total pools of these substances.

Thin Layer Chromatography (TLC)

Ten microliters of the PCA supernatant were applied without heat to a 2-centimeter strip of an Eastman Kodak Cellulose Thin Layer Chromatogram #13255 with a mylar backing, and developed with a mixture of water: formic acid: tertamyl alcohol: 1:2:3 for 5 hours. The chromatogram was dried in air, cut into 15, 1-centimeter strips, and each placed in a 5-milliliter counting vial with 3 mℓ of scintillation fluid. The vials were counted in a scintillation spectrometer and each count calculated as a percentage of the total U-14-C nucleotide pool. A typical representation of the toxic effects of m-xylene is seen in Figure 29 with substantial decreases in ATP and ADP and an increase in AMP. Significantly greater amounts of unmetabolized adenine are seen in the cells exposed to m-xylene. Recovery is 95-102%. In a large series of normal platelets carried out in connection with other projects ATP was 65-70%, ADP was 10-14% and AMP was 1-5%. Changes of 15% or larger in ATP levels were regarded as being above the analytical and biological noise levels for this determination.

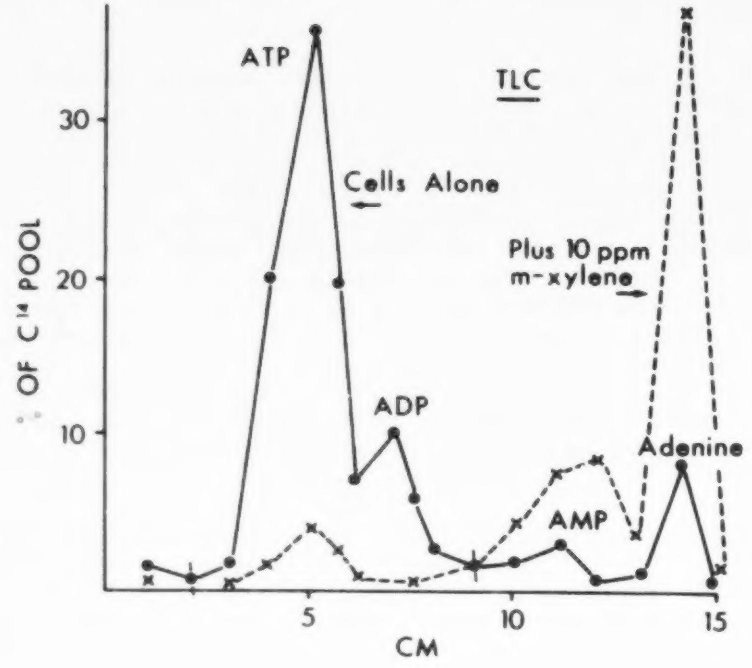


Figure 29. Toxic effects of m-xylene on platelets.

High Performance Liquid Chromatography (HPLC)

The major technical advance which makes it feasible to consider on-line capability is the use of high performance liquid chromatography (HPLC) for the rapid determination of the pool of adenine nucleotides at the rate of 15 minutes per sample. The peak heights are measured and used for calculation of pool size. Both TLC and HPLC are sensitive down to the 10^{-9}M range (15).

A Waters & Associates HPLC apparatus with a U-18 micro-Bondapak reversed phase column was used. Fifteen microlilters extract was injected and 0.1 M KH_2PO_4 was pumped through the column at 1.5 mℓ/minute. The nucleotides and related compounds were adequately separated in 12-15 minutes and were detected by UV absorption at 254 nm (see Figure 30). Identification was

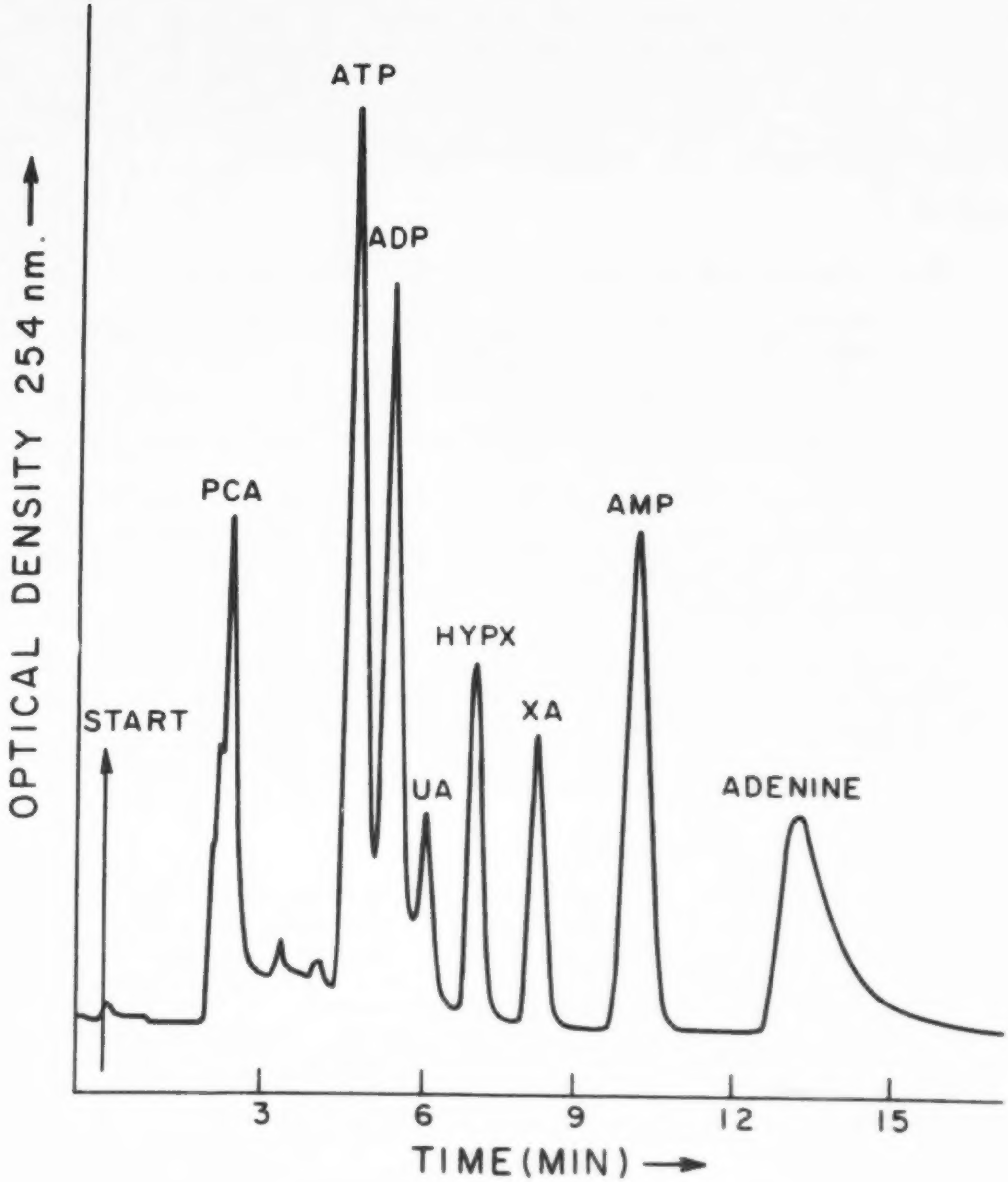


Figure 30. Elution curve of pure standards using HPLC.

obtained using retention time and the addition of known compounds to the test PCA supernatant. In the future it is planned to pump the effluent from the HPLC system into a flow-through scintillation spectrometer so that specific activity of each compound and be computed, thus obviating the need for TLC. Recovery and reproducibility of the standards was 95-103%. The peak height was found to be linearly proportioned to concentration, regardless of peak width, in the range 10^{-4} to 10^{-9} moles or purine compound per sample injected.

RESULTS

The results are divided into three main sections:

1) Influence of known toxic substances on platelets, neutrophils, and monocytes.
2) Effects of concentrated fractions of water on neutrophils and monocytes.
3) Study of reverse osmosis permeate and carbon treatment.

The chemical fractionation of various waters was carried out by Dr. Walton and is fully described in his section of this report. In order to avoid drawing up numerous tables, graphical representation was used whenever possible and the numbers printed on the graphs.

A list of the substances tested is shown in Table 7. For comparison of the cellular results with gross toxicity, the Repository of Toxic Effects of Chemical Substances (RTECS, compiled by NIOSH) was used to compile the exposure standards and toxicities of these compounds (Table 8).

Effects of Known Substances

Known compounds with the highest available grade of purity were added to the cell suspensions as described above. Initially, measurements were made of U-14-C adenine incorporation into ATP, ADP, and AMP, using TLC. Later on, when HPLC became available, the total adenylate pool was measured. A control with no additive was run with each batch of cells. Metabolic stress effects are indicated, either by a reduction in the 14-C or 12-C ATP pools (Table 9). Platelets, neutrophils, and monocytes were used for testing the toxicity of the known substances and are discussed below.

Platelets--

Figure 31 shows significant reductions from normal for U-14-C ATP in platelets treated with a range of chemicals, all at 2 ppm. In extrapolating from these results to gross toxicity data available in RTECS, we found that certain correlations exist. For instance, although the correlation is not perfect, chloroform is more toxic than toluene in both the cellular ATP

TABLE 7. KNOWN SUBSTANCES TESTED ON THE 3 CELL TYPES

	Cell Type		
Test Substance	Platelets	Neutrophils	Monocytes
Chloroform	+	+	+
Toluene	+	+	+
1:1:1 Trichloroethane		+	
1:2 Dichloroethane		+	
o-dichlorobenzene		+	
Tetrachloroethylene	+	+	
Hexanal		+	+
m-Xylene		+	+
p-Xylene	+		
Methanol		+	
n-Octane	+		+
Trichlorethylene	+		+
N-Hexane	+		

TABLE 8. GROSS TOXICITY DATA FOR HUMAN AND RAT EXPOSURE*

Substance	Human Work Std. (ppm) (NIOSH)	LD50 mg/kg (rat)
Chloroform	10	800
0-dichlorobenzene	50	520
p-dichlorobenzene	75	500
Dimethylsulfide	-	535
1,2-Dichloroethane	50	680
Carbon tetrachloride	-	1770
Trichlorethylene	100	4920
Tetrachloroethylene	100	5671
Hexanal	-	4890
Toluene	200	5000
Ethylene dichloride	200	5750
o,m-, & p-xylene	-	(dog) 5000
Trichloroethane	350	9470
n-octane	500	-
n-Hexane	500	-

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*Repository of Toxic Effects of Chemical Substances, NIOSH USDPH Rockville, Md., 1976.

TABLE 9. RESPONSE OF NEUTROPHILS AND PLATELETS TO VARIOUS PURE COMPOUNDS AND TO FRACTIONS OBTAINED FROM DRINKING WATER

	Added conc. ppm	% $\frac{C^{14}\ \overset{TLC}{\overline{ATP}}\text{ of}}{C^{14}\text{ pool}}$	$C^{12}\ \overset{HPLC}{\overline{+\ C^{14}}}$ ATP peak ht cm	Cell
Hexanol	0.0	50.0	-	Neutrophil
	0.1	4.1	-	
	1.0	0.0	-	
	10.0	0.0	-	
Trichloroethylene	0.0	62.0	11.0	Neutrophil
	0.01	0.0	4.2	
	0.10	0.0	2.1	
	1.00	0.0	3.5	
	10.00	0.0	1.4	
Toluene	0.0	61.0	10.9	Neutrophil
	0.1	63.0	12.2	
	1.0	66.0	12.6	
	10.0	5.3	0.4	
Metaxylene	0.0	57.0	8.6	Platelet
	0.1	63.0	9.6	
	1.0	61.0	8.5	
	10.0	6.2	0.0	
Chloroform	0.0	59.0	4.5	Neutrophil
	0.1	60.0	4.5	
	1.0	45.0	2.7	
	1.3	28.0	-	
	2.6	17.0	-	
	10.0	2.9	0.7	

(continued)

TABLE 9 (continued)

	Added conc. ppm	TLC % C^{14} ATP of C^{14} pool	HPLC C^{12} + C^{14} ATP peak ht cm	Cell
Napthalene 2-methoxy, 6-isoproprionic acid	0.0	-	23.0	Platelet
	0.1	-	21.4	
	1.0	77.0	21.8	
	10.0	79.0	20.8	
2-bromo 6 methoxy napthalene	0.0	-	23.0	Platelet
	0.1	63.0	24.5	
	1.0	74.0	17.9	
	10.0	61.0	16.0	
2-methoxy-naphthalene	0.0	-	23.0	Platelet
	0.1	76.0	21.6	
	1.0	68.0	22.7	
	10.0	74.0	20.5	
n-octane	0.0	72.0	-	Neutrophil
	20.0	63.0	-	
n-hexane	0.0	72.0	-	Neutrophil
	20.0	25.0	-	
111-trichloroethane	0.00	70.0	-	Neutrophil
	0.10	17.0	-	
	1.00	13.0	-	
Dichlorobenzene	0.00	71.0	-	Neutrophil
	0.01	68.0	-	
	0.10	66.0	-	
	1.00	72.0	-	

(continued)

TABLE 9 (continued)

	Added conc. ppm	% C^{14} ATP of C^{14} pool TLC	$C^{12} + C^{14}$ ATP peak ht cm HPLC	Cell
Cincinnati drinking water concentrate (Supplied by HERL, EPA)	0.00	76.0	17.2	Platelet
	1.40	62.0	15.3	
	1.40	-	11.7	
Fraction G4 Secondary Effluent Pomona	0.0	51.0	10.0	Neutrophil
	1.1	5.8	0.0	
Negative Controls				
(i) Dimethyl sulfide	0.0	55.0	11.0	Platelet
	12.5	53.0	10.6	
	25.0	45.0	10.3	
(ii) Glucose	0.0	56.0	13.0	Platelet
	600.0	54.0	13.6	
	1200.0	56.0	13.5	

response and the qualifying toxic dosages. Similarly, octane is less toxic than toluene.

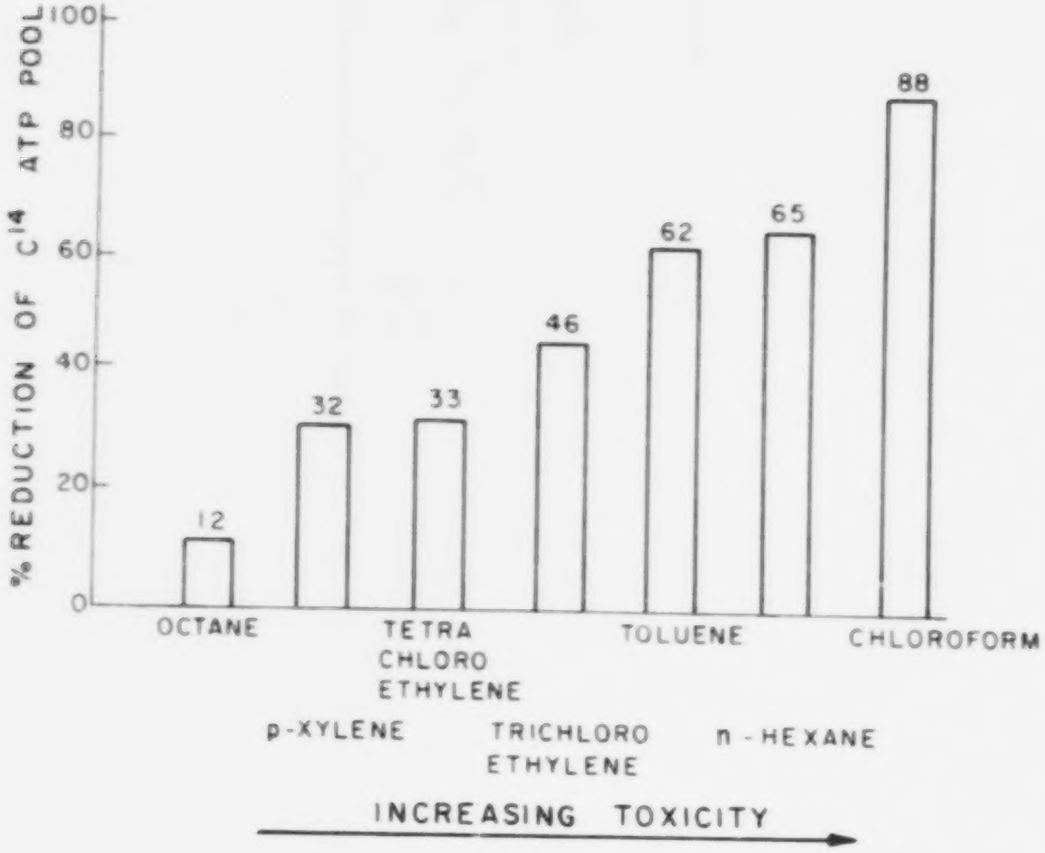

Figure 31. Effects of known substances at 2 ppm on U-14-C ATP pools of platelets using TLC.

Dimethyl sulfoxide (DMSO) and glucose were included as negative controls. DMSO is used for cell and organ preservation and has been given in large amounts to patients with scleroderma. DMSO is also used to solubilize the constituents of water concentrates. Glucose is also ingested in large quantities in our diet and is not acutely toxic to cells over a large range in concentration (600-1200 ppm) unless insulin or glucogen metabolism is disturbed. Our results showed that these two materials were without significant effect on the metabolic parameters being used as criteria for toxicity (Table 9), thus lending increased validity to the usefulness of these tests for establishing toxicity criteria.

Although there are many factors which influence the toxic effects of a chemical, the broad correlation which is present indicates that cellular ATP measurements may not be too far removed from clinical toxicity effects. Although this work was concerned with ATP because of the universality of its function in living systems, one need not be restricted to this pathway in future work.

Neutrophils--

Rankings of the toxic effects of the chemicals acting on neutrophils at the dosage levels of 0, 0.1, 1.0, and 10 ppm of substance added is shown in Figures 32, 33, 34, and 35. As with platelets, neutrophils respond to varying concentrations of these compounds, still preserving the main features of the RTECS ranking. The dose response characteristics are shown in Figure 35. Chloroform and trichloroethane give significant responses at 0.1 ppm, whereas toluene, xylene, 1:2 dichloroethane and o-dichlorobenzene affect these cells at the 1 ppm level.

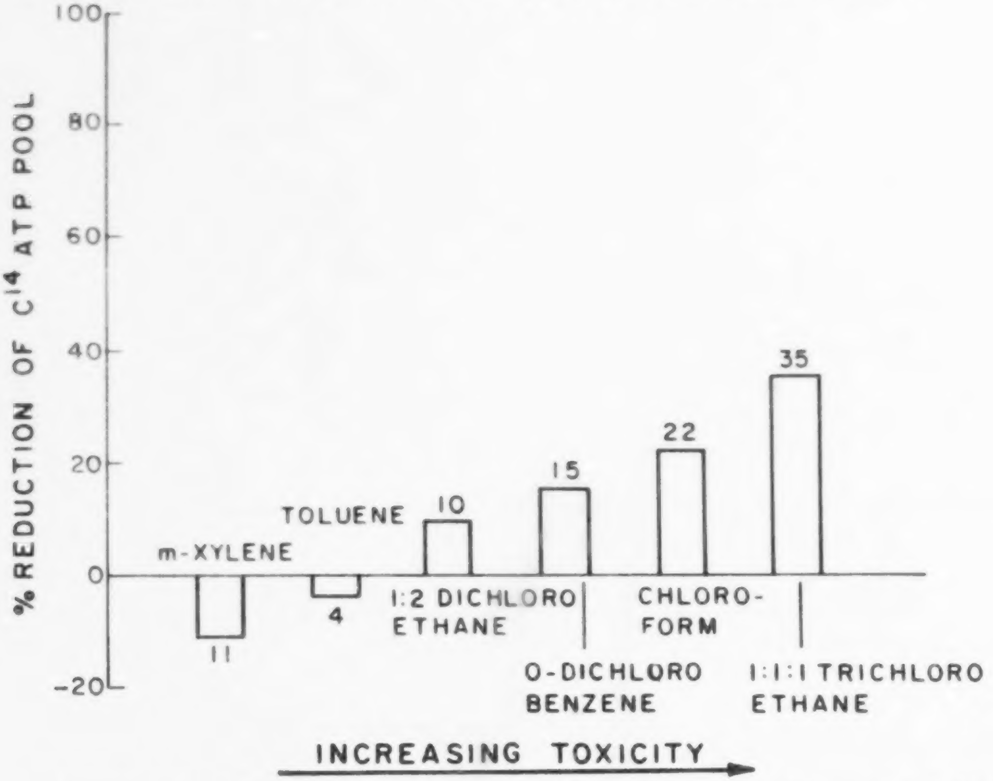

Figure 32. Effect of known substances at 0.1 ppm on the U-14-C pool of neutrophils by TLC.

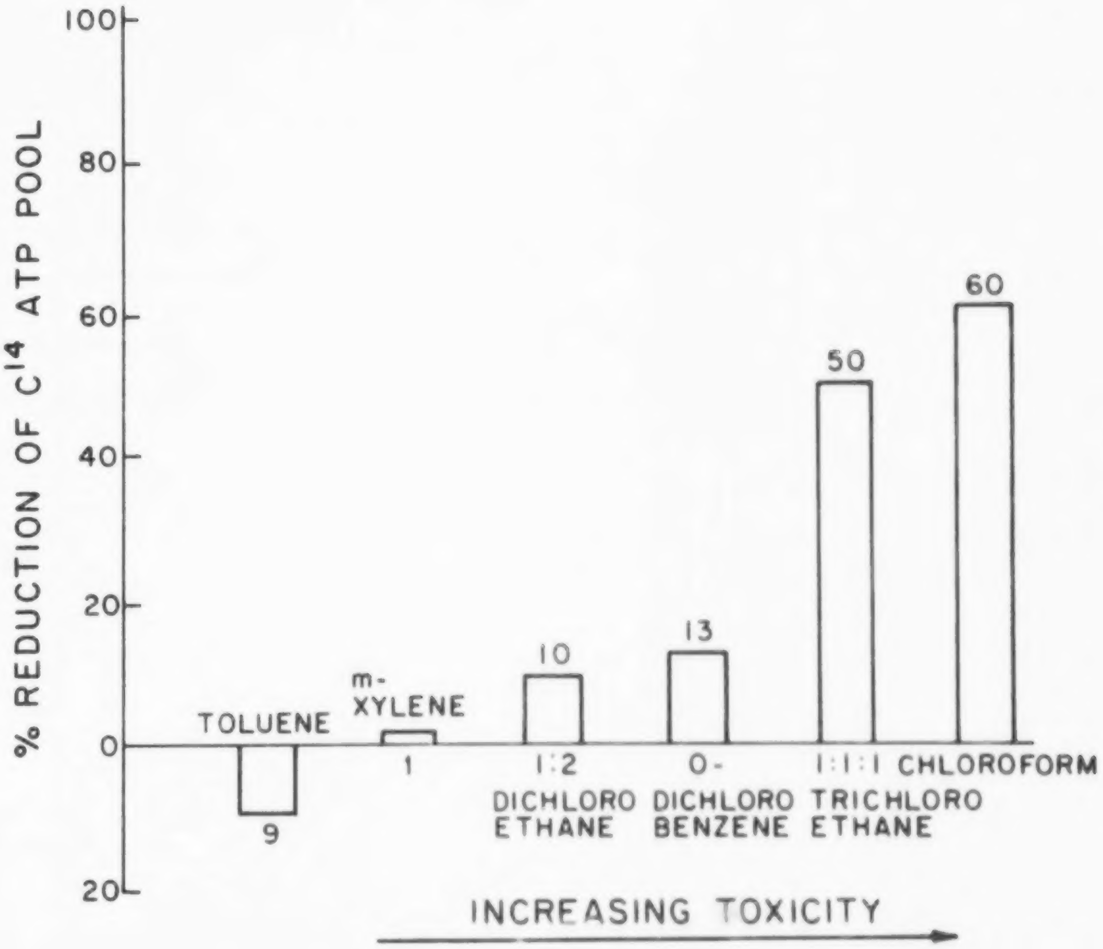

Figure 33. Effect of known substances at 1.0 ppm on the U-14-C ATP pool of neutrophils by TLC.

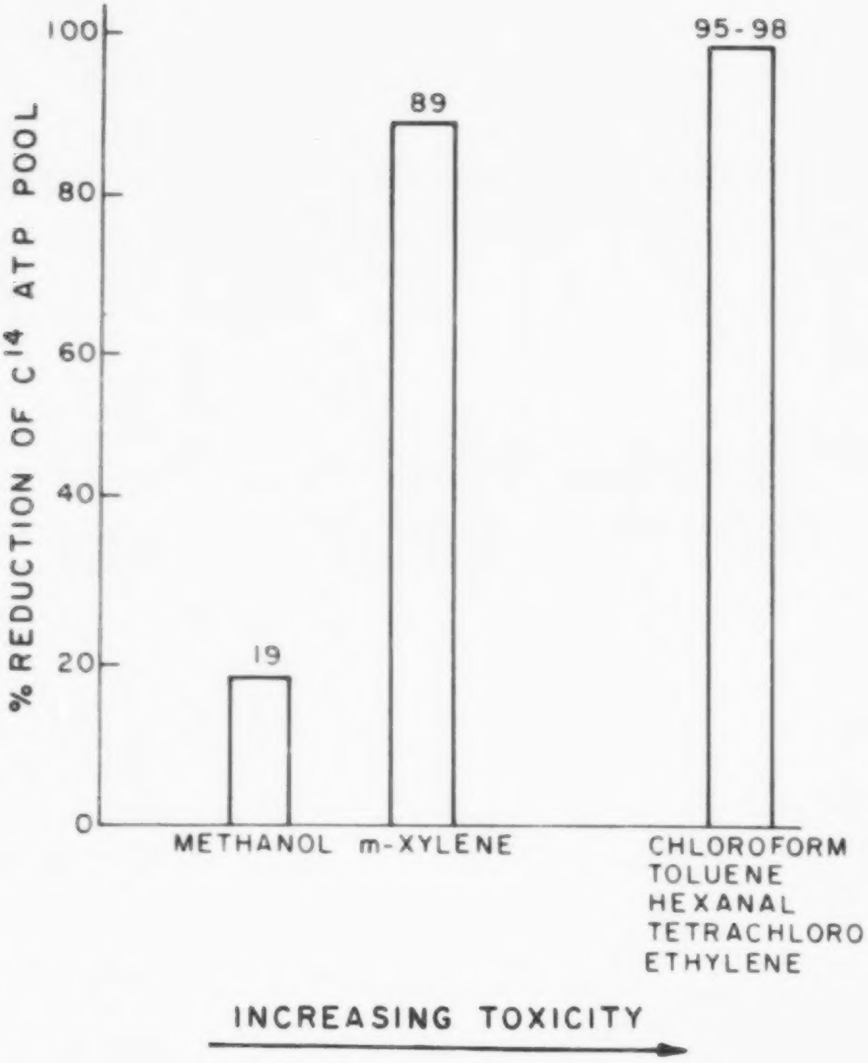

Figure 34. Effect of known substances at 10 ppm on the U-14-C ATP pool of neutrophils by TLC.

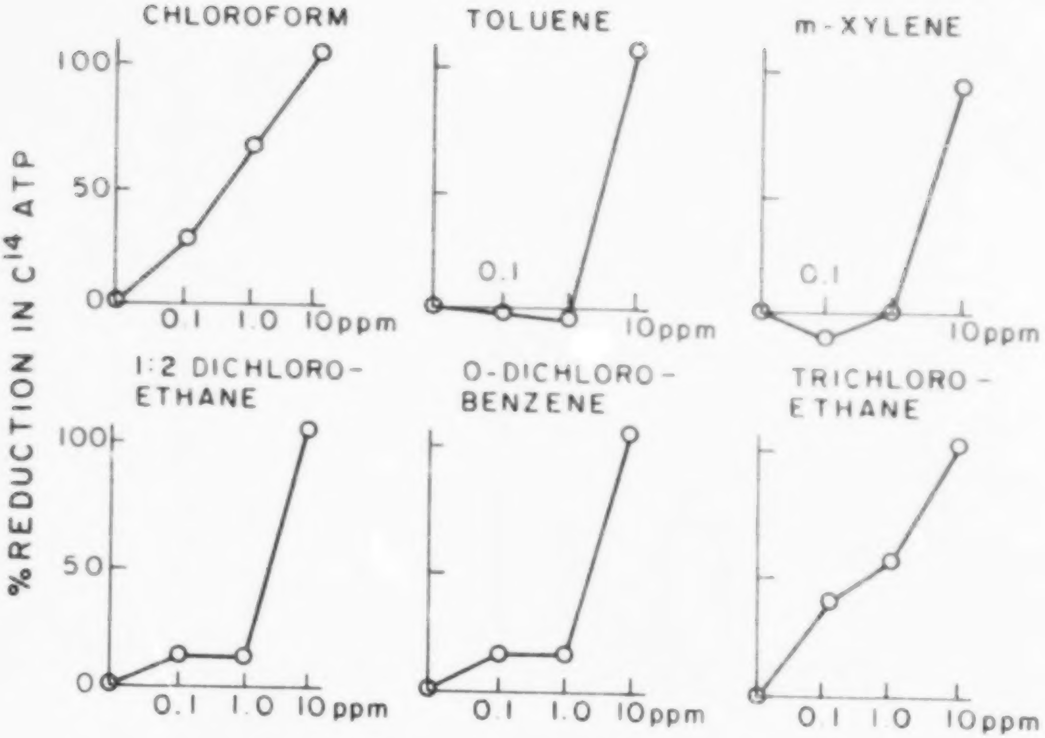

Figure 35. Dose response curves of pure substances acting on neutrophils.

Monocytes--

Monocytes were allowed to attack red cells labeled with radioactive chromium and the killing power was determined by measuring the chromium release. The same cells were examined for changes in U-14-C ATP and total ATP pool using both TLC and HPLC. The results were compared with each other at 0.1, 1.0, and 10 ppm of added known substances (Figures 36, 37, 38, 39). In general, the TLC and HPLC dose response curves were in good agreement (Figures 39,40). The HPLC curves often showed a more sensitive response at the 0.1 and 1 ppm levels. The monocyte, however, was found to be most resistant to chloroform at concentrations of 0.1 and 1.0 ppm, responding at 10 ppm. Except for tetrachloroethylene the monocyte did not give a graded response for target cell release of chromium with increasing concentration. Thin layer chromatography and HPLC were more generally sensitive in detecting graded cell responses. These results were most satisfactory from a technical viewpoint and large numbers could be processed per day. However, interpretation is more difficult with regard to RTECS ranking because of the refractory behavior of the monocytes to 1 ppm of chloroform which ranks high in toxicity and because of their larger response to compounds such as m-xylenes and p-xylenes, which have lower overall toxicities than chloroform. Nevertheless, it is important to realize that a chemical which is relatively low in gross human toxicity may have a considerable effect on the activity of this important cell. In all the cells a concentration of added substance at 10 ppm was too high for the discrimination between individual compounds, which is necessary for ranking. Ranking was more efficient at the 1 ppm level of exposure.

Effects of Concentrated Water Fractions on Neutrophils and Monocytes

Concentrated fractions were produced and labeled from G1 to G9. The higher the G number the lower the polarity of the constituents of the fraction. Figure 34 shows the response of neutrophils to dilutions of each fraction. Due to the unavailability of reliable total organic values, an absolute concentration is not given for each fraction. Nevertheless, very significant responses were obtained with toxicity increasing with the G number of the fraction. Thus, G2 is seen to be relatively non-toxic to neutrophils, but G4, G5 and G9 produce significant reductions in the U-14-C ATP pool.

With regard to monocytes very similar results were obtained compared to those obtained on neutrophils (Figure 41). These results on extracts of Pomona, California wastewater note the changes in chromium release and U-14-C ATP pools. As before, toxicity generally increases with G number. Of special interest is G4 and G4A; G4A is charcoal-treated in the plant and by comparison with the untreated G4 fraction it is seen that

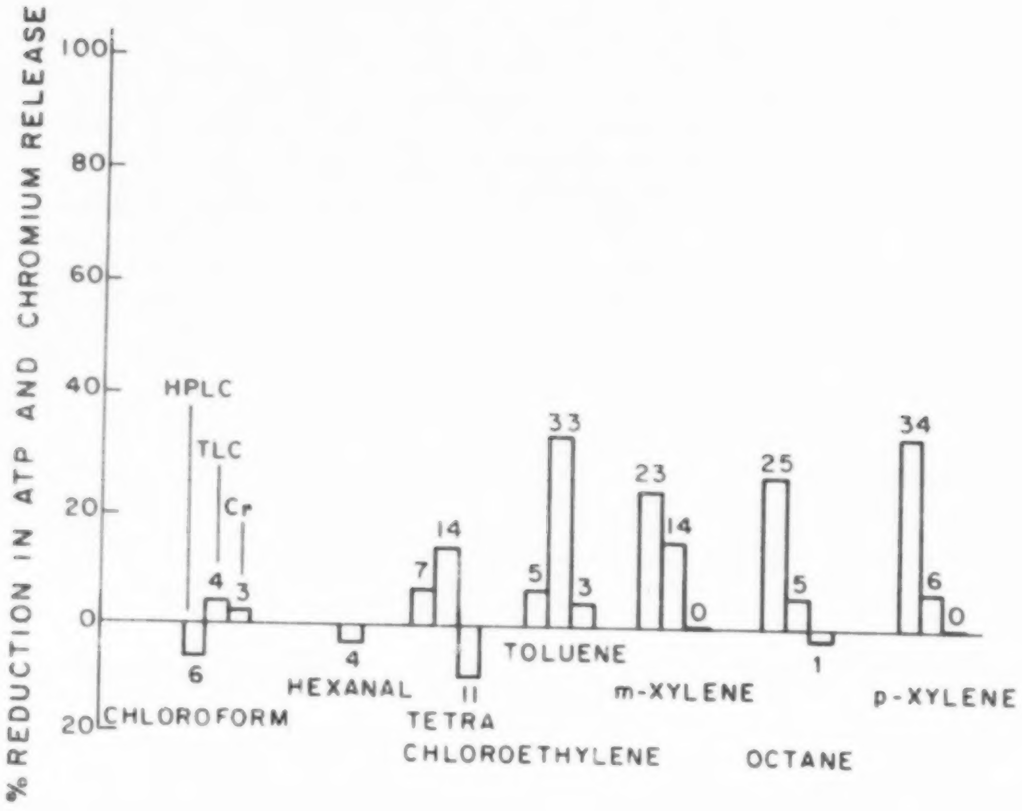

Figure 36. Effects of known substances at 0.1 ppm on the U-14-C ATP and total ATP pools in monocytes.

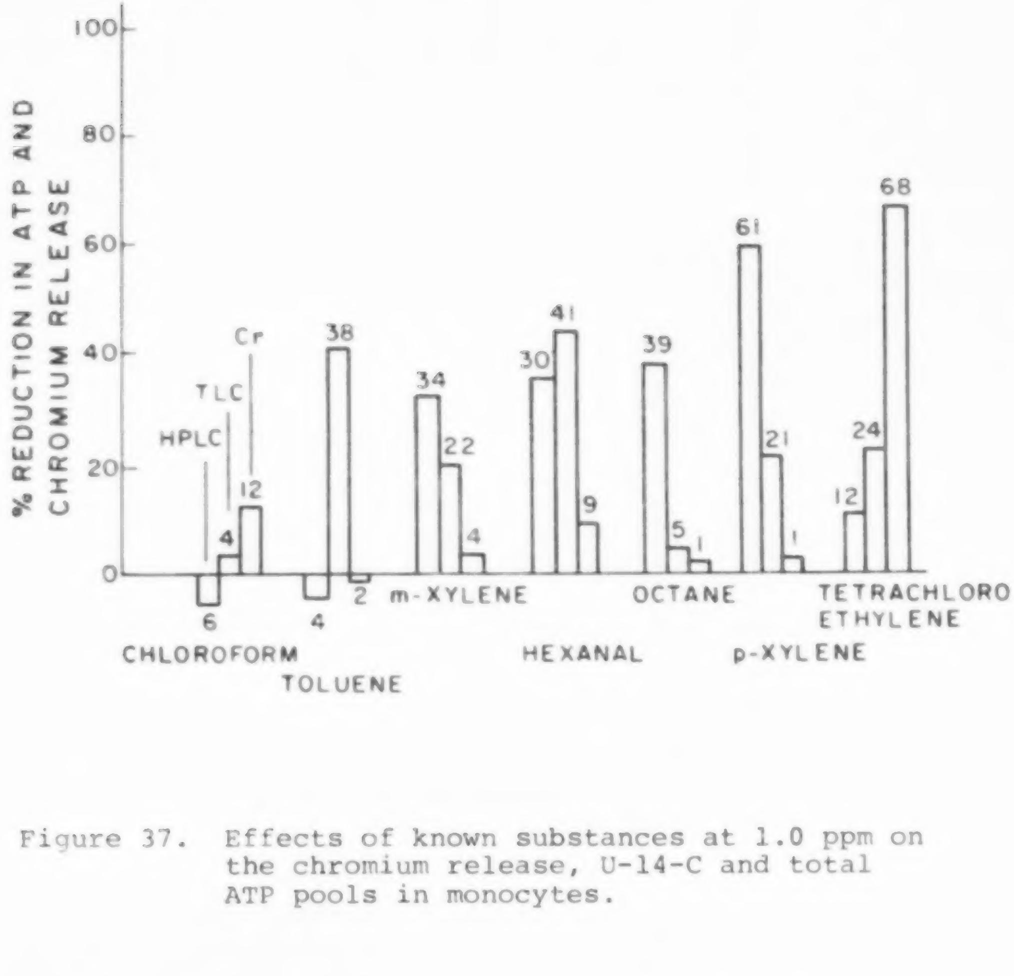

Figure 37. Effects of known substances at 1.0 ppm on the chromium release, U-14-C and total ATP pools in monocytes.

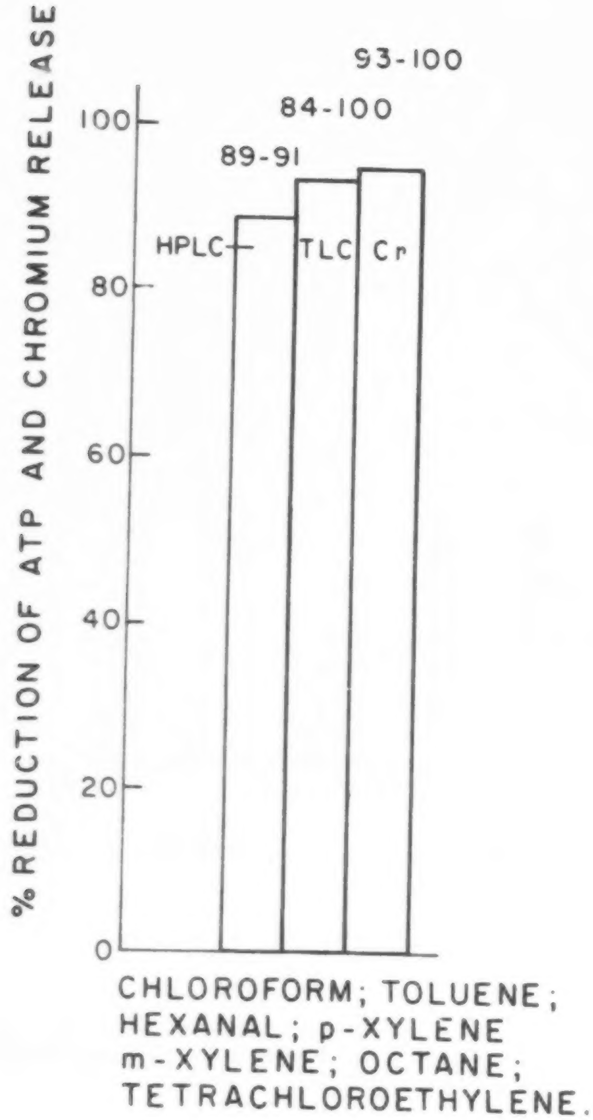

Figure 38. Effects of known substances at 10 ppm on the chromium release, U-14-C ATP and total ATP pools in monocytes.

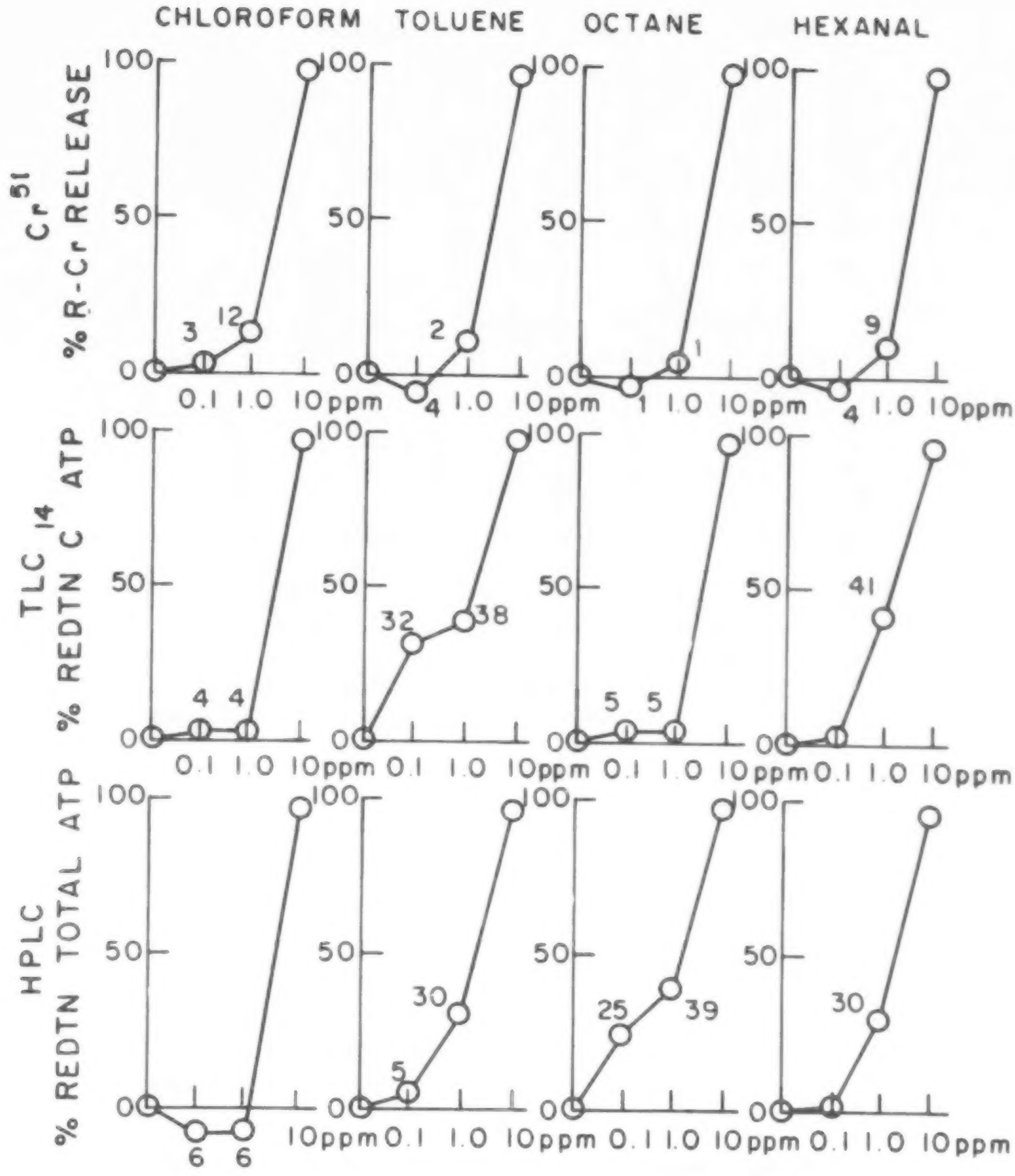

Figure 39. Dose response curves of pure substances in contact with a suspension of monocytes, 0-10 ppm. (continued)

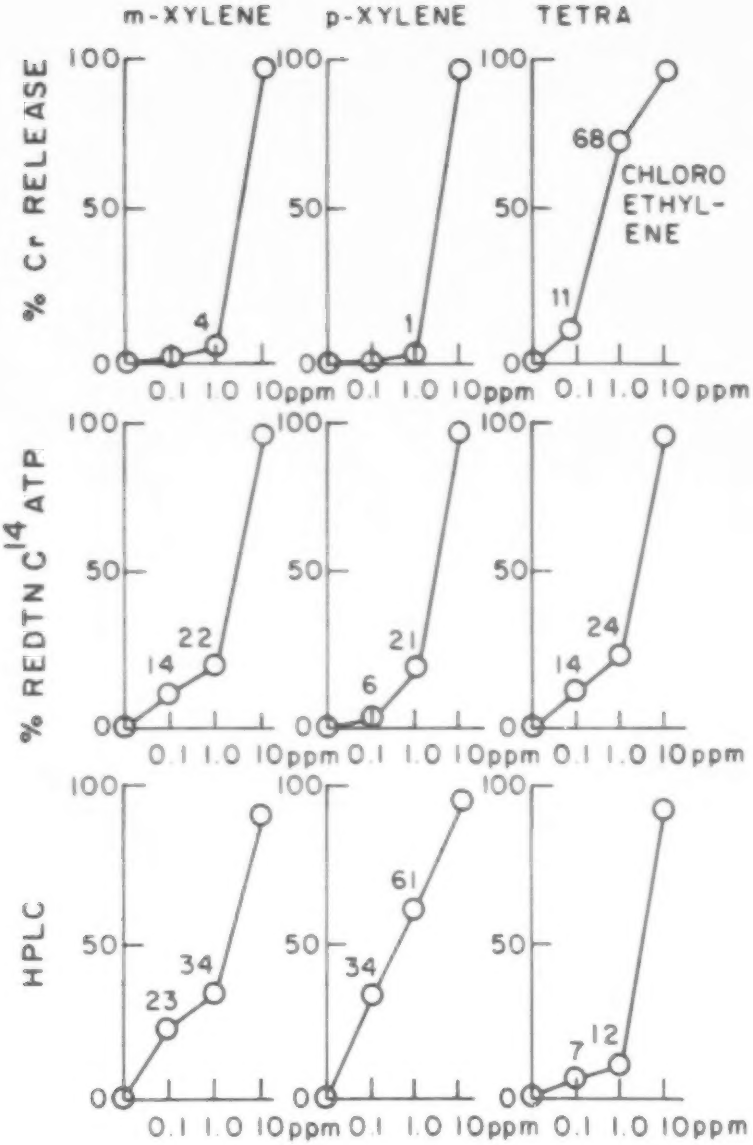

Figure 39 (continued). Dose response curves of pure substances in contact with a suspension of monocytes, 0-10 ppm.

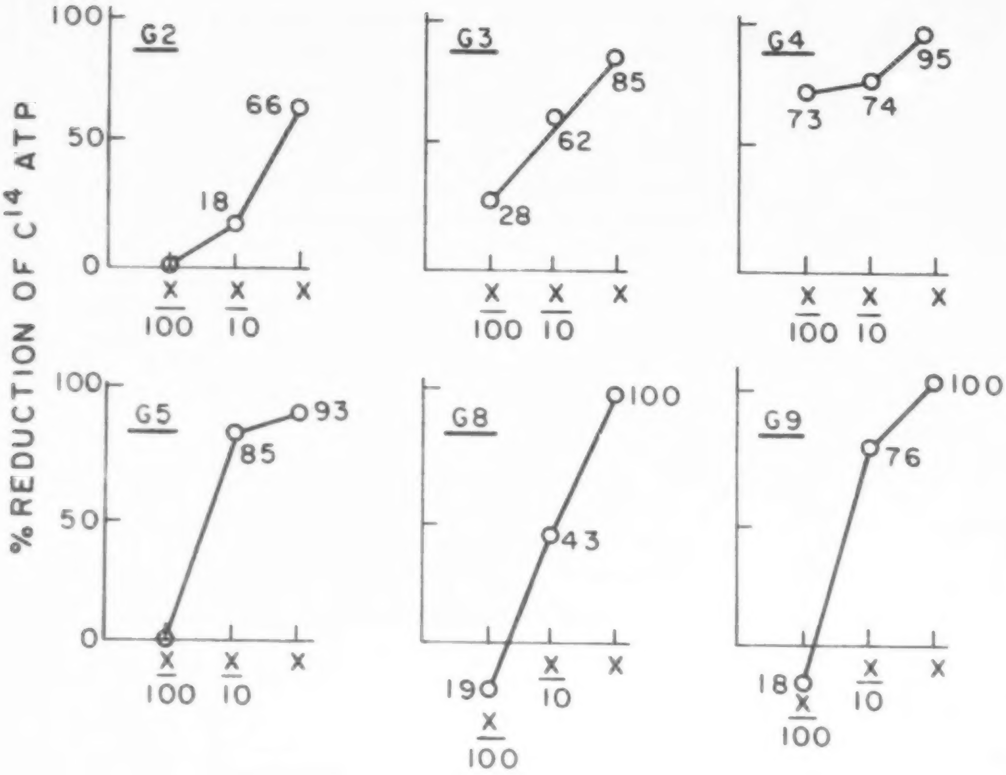

Figure 40. Dose response of the U-14-C ATP pool to concentrated extracts of sewage; TLC polarity of the constituents decreases as G increases.

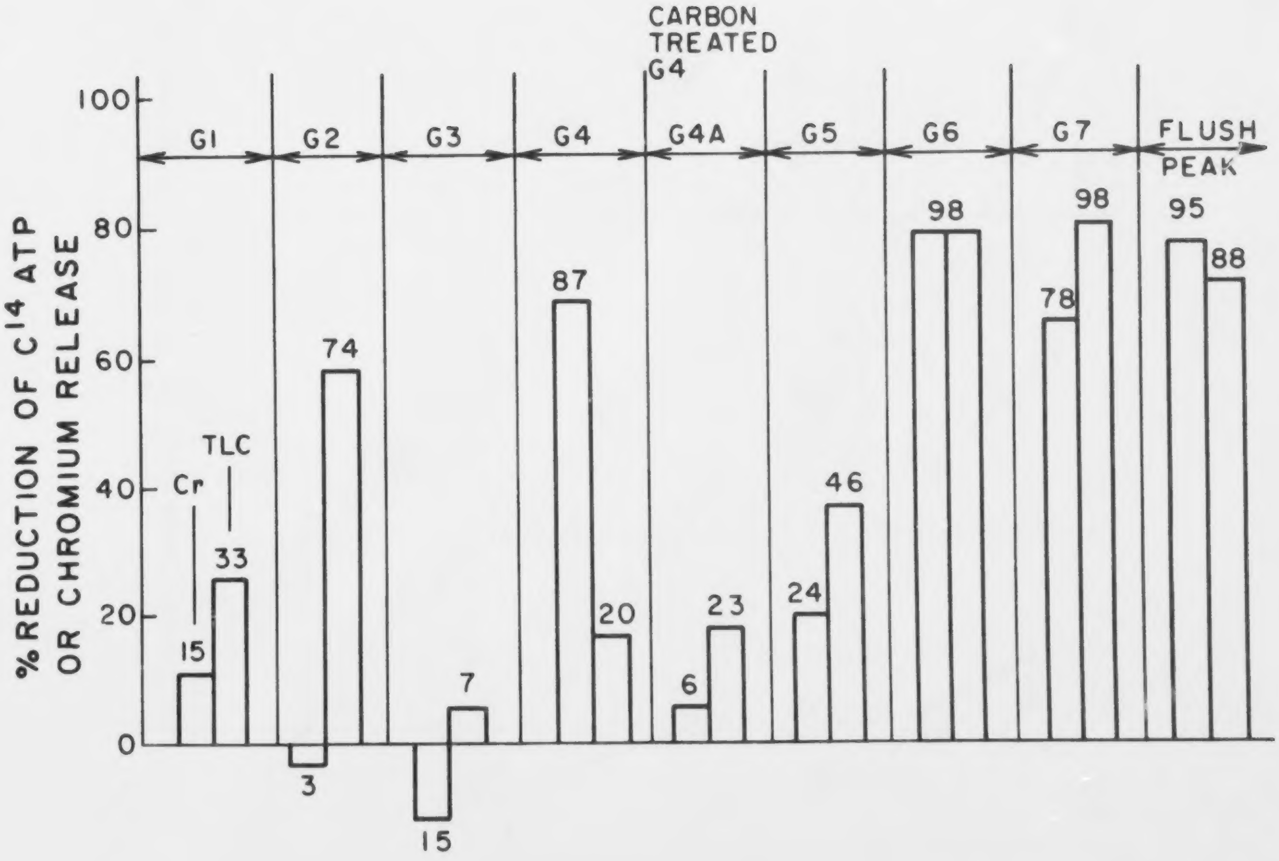

Figure 41. Comparative toxicities of fractions from Pomona, California wastewater using TLC and chromium release using monocytes. Each fraction was diluted 1:1 before use.

toxicity is greatly reduced by this treatment. Thus, it may become feasible to monitor separate unit processes within a treatment plant by means of our tests and pinpoint the entrance and removal of toxic materials.

Reverse Osmosis

Our toxicity methodology is readily adaptable to the study of pilot plant or laboratory scale purification processes. In this regard G3 and G4 concentrates were obtained from laboratory reverse osmosis apparatus. Both the permeate and reject of sewage were tested using neutrophils. As before, using the criteria relating to ATP metabolism, G3 permeate was less toxic than G4 and the reject, as expected, was highly toxic (Figure 42).

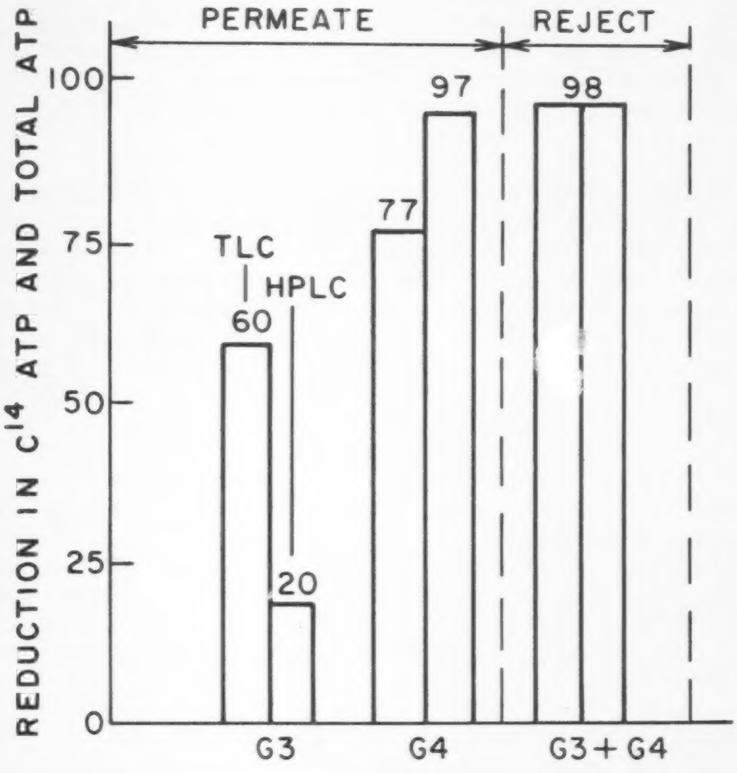

Figure 42. Reverse osmosis; toxicity of G3 and G4 permeate and reject fractions using neutrophils.

DISCUSSION

In the space of three years we have progressed from a theoretical understanding of stress effects in human cells to a practical application to water extracts and the effects of unit processes such as charcoal treatment and reverse osmosis.

The utilization of cell incubation followed by HPLC and TLC analysis offers considerable capability for the sensitive and rapid detection of cellular toxicity. The system is flexible in that it is not limited to any particular metabolic pathway or cell type and is relatively inexpensive. The system is vulnerable in that it needs a constant supply of normal cells of organisms. However, continuous 5-point dose response curves can be obtained using platelets from 10 mℓ of blood per day available from a nearby blood bank. Alternatively, one could use in vitro cell cultures of fibroblasts or bacteria which can be made up in large batches. As long as sufficient cells are present an accurate cell count is not needed, provided the same suspension is used for test and control. If necessary, a portion of the cells can be diverted for functional studies or other investigations.

Progress was also made with regard to three previously mentioned problems of sensitivity of the assay, limited exposure time, and the selection of a model pathway. In general, sensitivity was adequate down to the 0.1 ppm range for known compounds, and water concentrates of approximately 800 fold. It is estimated that by increasing the ratio of test substance to cells, an improvement of at least 10-fold could certainly be obtained and longer times of incubation could probably improve this by an addition factor of 2-5. With regard to the selection of a metabolic pathway, it is true that certain types of toxicity would be missed, for example, mutagenicity and very specific enzyme effects. Nevertheless, the adenylate pathway is of central and unique importance in the transduction of energy and the kinetic control of metabolism. Thus, a major disturbance here would have serious consequences for any cell. More specific tests of toxicity could be done in response to a detailed knowledge of the chemical constitution of the components of the water concentrate.

In general, reductions in ATP as determined by HPLC and TLC are detectable for most compounds present at concentrations of 0.1 ppm, with larger effects found at 1.0 ppm. At this order of concentration it was possible to rank the substances in order of cellular toxicity. At the higher exposures of 10 ppm the cellular toxicity was so great as to render distinction between compounds virtually impossible.

Platelets have their limitations with respect to the fact that they lack a nucleus and its DNA. For this reason, other cells need to be used when mutagenic characteristics are of interest. However, no serious problems are forseen for the practical adaptation of these cells for continuous surveillance of metabolic toxicity of water effluents. The availability of platelets from small amounts of blood (1-5 mℓ/dose response curve), their stability and their quick response to perturbation makes them extremely suitable for our purpose. They are remarkably stable if kept suspended in their original plasma and not subjected to mechanical trauma. White cells (neutrophils) were more prone to deterioration due to technical reasons, and preparations had to be discarded on several occasions. Monocytes were very stable but their responsiveness to toxic materials present in concentrations below 10 ppm was not great. Furthermore, the toxicity ranking differed from the gross human and rat toxicity found in RTECS listings. Nevertheless, the monocyte needs to be developed further because if its sensitivity can be improved, it has the potential for detecting toxicity in compounds that are presently not thought to be highly dangerous.

It is difficult to decide which of these three cell models is the most useful for monitoring purposes. They all have their advantages and disadvantages. With on-line operation ultimately of prime importance, the platelet is recommended because of its stability and ease of analysis with only small amounts of blood (1-5 mℓ) needed per dose response curve. On the other hand, neutrophils and monocytes tell us more about the immune defense system, while platelets do not. However, larger volumes of blood on the order of 50 mℓ are required and the behavior of the neutrophils may be subject to seasonal variations. The monocytes were less responsive overall and their toxicity ranking did not seem to follow the RTECS listings as well as the other cells. However, monocytes gave highly reproducible results.

In the first year considerable work was expended in simplifying the methodology, testing known compounds, and learning how to control the sources of variation. In the second year known substances continued to be tested, but the greatest emphasis was placed on testing fractions of treated sewage. In the third year a high performance liquid chromatography apparatus was purchased and it was then possible to include total adenylate pools in our toxicity assays as suggested by previous EPA reviews of our work. Many more fractions were analyzed and the results extended to a study of treated wastewaters from Pomona, California waste treatment plant. Distinct differences were noted in the effects of this water before and after charcoal treatment. Both toxic and non-toxic fractions were found in the samples. Thus, the methodology had sufficient range to distinguish biologically between fractions of different chemical polarity. The humic acid fraction was non-toxic, but less polar compounds had adverse effects on the cells.

SUMMARY AND CONCLUSIONS

The main objectives of the testing were met with regard to the items listed below:

1) Several model systems for measuring cellular toxicity were developed using platelets, neutrophils, and monocytes.
2) Testing included known substances, water fractions, and concentrates from sewage and water treatment plants. Degrees of toxicity and the effects of treatment were measured.
3) The metabolic pathway of adenylate metabolism was found to be suitable as a rapid method of analysis and allows for a unified, theoretical approach to toxicity of a wide variety of compounds.
4) Correlation of cellular toxicity with gross toxicity quoted in the literature was satisfactory for platelets and neutrophils.

SECTION 5

TOXICITY TESTS: CELLULAR BACTERICIDAL STUDIES

INTRODUCTION

The objectives of the work reported in this section can be summarized as follows: to develop functional assays that will indicate whether the specialized activities of white blood cells have been compromised by exposure to environmental substances.

White cells were selected for study because of their important function in protecting the body against invasion by bacteria and in combating malignancy. The cells carry out their bacteriocidal functions by first sensing the presence of the bacteria moving to the site of infection, ingesting the bacteria (phagocytosis), and then killing them chemically by metabolic action. Emphasis was placed on the phagocytosis and killing power assays of neutrophils and monocytes. Difficulties related to contaminating red cell removal were overcome by a modified lysis technique of short exposure to ammonium chloride; further stabilization was effected by suspending the cells in tissue culture fluid RPMI-1640 to which 10% fetal calf serum was added.

Despite these modifications the screening of large numbers of samples was impossible since the complexity of the neutrophil technique allowed for only 3 or 4 assays per day. This problem became particularly acute as Drs. Walton and Eiceman improved their techniques of separation of compounds from water, resulting in a large number of peaks to be tested from an individual water sample. In order to do dose response curves on individual fractions, this technique severely limited the number of samples that could be analyzed at one time. Fractions from a single water sample could be analyzed at one time. Fractions from a single water sample would have to be analyzed over a two week period, and the sensitivity of the bacterial assays for phagocytosis and killing power were relatively low. Fortunately this problem was overcome by the use of human peripheral blood monocytes in a new assay of human cellular function.

The monocyte assay was first introduced in late 1976 (16) and was given the name antibody dependent cellular cytotoxicity (ADCC). It required both human antibody and human cells to destroy chromium-labeled target cells. It was soon discovered that ADCC was an essential function in the elimination of cancer cells and the termination of viral infections. The human ADCC system has the major advantage of being able to screen several hundred samples per day. An outline of the methodology used for the neutrophil and monocyte assays is given below. Table 10 shows the substances tested.

TABLE 10. KNOWN SUBSTANCES TESTED BY NEUTROPHIL AND MONOCYTE ACTIVITY

1:1:1 Trichloroethane
1:2 Dichloroethane
Tetrachloroethylene
o-Dichlorobenzene
Chloroform
Dimethyl sulfide
Trichloroethylene
n-Octane
m-Xylene
p-Xylene
n-Hexanal
Toluene
Carbon tetrachloride

METHODOLOGY AND RESULTS

Known Substances

Neutrophil Testing--

The neutrophils were obtained by separating anti-coagulated blood immediately after venipuncture using a column of methyl cellulose hypaque. Contaminating red cells were lysed with ammonium chloride (0.85 gm/100mℓ).

After obtaining the white blood cell suspension and adjusting the cells to the desired concentration of approximately 10,000/mm^3, the cells were incubated for 30 minutes at 37^o in the presence of the test compounds. The cells were then washed twice by centrifugation and resuspended in tissue culture medium RPMI-1640 with antibiotics and 10% fetal calf serum, and assayed for phagocytosis and killing power. A portion of the cells was immediately taken to Dr. Solomons' laboratory in the same building for ATP metabolism studies.

In order to measure phagocytosis and killing power the cells were exposed to a standarized number of bacteria (staphylococcus aureus 502A) and opsonis (8% AB serum) and incubated for another two hours at 37^o on the tilting table. This interval of time allowed for phagocytosis and killing of the bacteria by the neutrophils (PMN). After the incubation period the total number of bacteria surviving the experiment was determined by plating, and expressed as a percent of the number of bacteria present at the beginning of the two hour phagocytic assay. This value represents the interaction of the phagocytic process by PMN's and the killing of the bacteria by intracellular bactericidal mechanisms. Intracellular surviving bacteria were also determined in each assay using lysoraphin to eliminate cellular bacteria. This value correlates inversely with the PMN bactericidal activity. Finally, the number of extracellular bacteria (EB) was calculated for each experiment. The EB value correlates inversely with the phagocytic uptake of bacteria by the PMN's. Thus, phagocytosis is determined as the percentage of bacteria remaining outside the cells after exposure to the neutrophils. Killing power is calculated as the percentage of intracellular bacteria surviving after ingestion by human neutrophils.

Instead of using bacteria, an assay using the phagocytosis of oil particles was developed in order to improve the sensitivity quantitation and speed of analysis. Oil red-o phagocytosis involves the internalization of di-iso-decyl phthalate in oil which is coated with E. Coli lipopolysaccharide and serum to optimize its ingestion. The oil particle contains the dye Red-o, which can be quantitated by spectrophotometry. Although this method is sensitive and correlated well with bacterial phagocytosis, it requires too much blood to be practical for daily use.

Dichlorethane, tetrachloroethylene, and trichloroethane at 10 ppm significantly reduced neutrophil phagocytosis (Figure 43). Insignificant effects were observed with water fraction concentrates, G1, G2, G3, and G4, but G5, G8, and G9 showed measurable decreases in phagocytosis (Figure 43). However, the rest of the known substances had little effect. It was also thought that the additive could kill bacteria independently of the neutrophil, thus making interpretation difficult. Reverse osmosis permeate and reject did not have any significant effect on phagocytosis and killing power although marked effects were observed on ATP metabolism. Because of the relative insensitivity of this assay the monocyte assay system was developed to replace the neutrophil testing as described below.

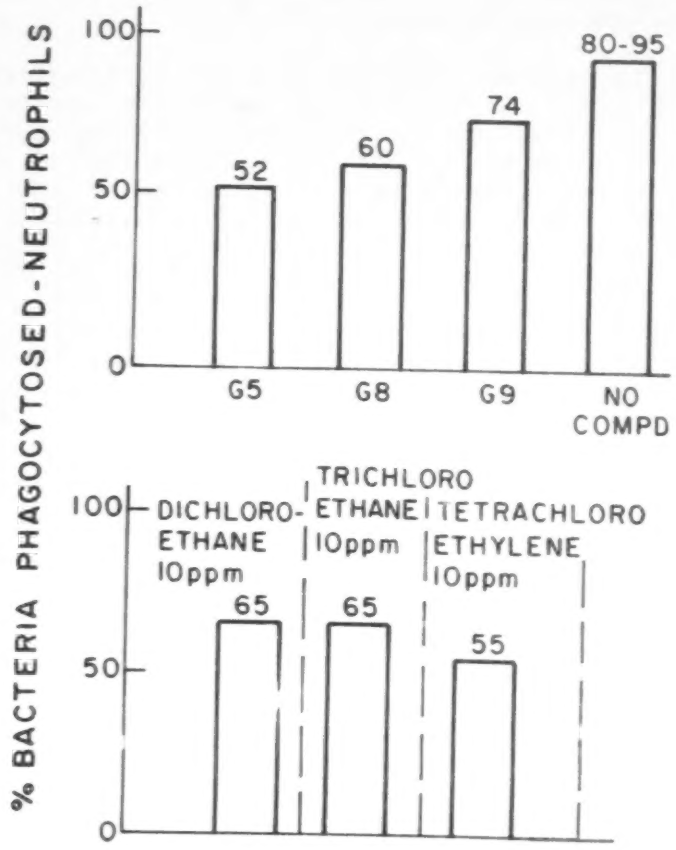

Figure 43. Effects of sewage fraction concentrates and known substances on phagocytosis using neutrophils.

Monocyte testing--

The Ficol Hypaque method was used to separate mononuclear cell populations from peripheral blood (17). Approximately 20-40% of the cells were monocytes, the rest being lymphocytes. After exposure to known toxic substances or concentrated water fractions for 30 minutes at 25°C the suspension was used as effector cells to attack chromium^{51}-labeled red cell targets which were coated with a specific antibody. The monocytes and lymphocytes recognize the Fc portion of the immunoglobulin molecules on the target cell and lysis is determined by release of the radioactive chromium label.

The results for monocyte testing are reported for both known substances and concentrated water fractions. Hexanal and tetrachloroethylene gave graded responses (Figure 44). The remainder of the compounds showed virtually no toxicity at 0.1 and 1.0 ppm but all had profound effects at 10 ppm. These substances included the following: chloroform, dimethyl disulfide, trichloroethylene, n-octane, m-xylene, p-xylene, toluene, and carbon tetrachloride. With regard to concentrated wastewater fractions from Pomona, toxicity generally increased as G number increased (Figure 45) in accordance with the results obtained on neutrophils (Figure 43) and the data on ATP pools (Figure 39). Carbon treatment (G4a) greatly reduced the toxicity of G4, confirming the results of the metabolic determinations.

DISCUSSION

The first year of this grant began with the development of the human neutrophil system as an assay system for water toxicity. Initial results were encouraging in that toxicity could be detected with the human neutrophil assay. However, experience over the next year led to two major difficulties:

1) Correlation of the human neutrophil bactericidal activity with human neutrophil metabolic activity was poor 85% of the time. This was predominantly due to the insensitivity of the human neutrophil bactericidal system.

2) The number of samples that could be screened at one time using the human neutrophil system was limited. Only three or four samples could be assayed in one day. Although a considerable number of assays of toxic chemicals and water concentrates were performed using the human neutrophil assay system, this limitation meant that screening of large numbers of samples was impossible.

The human ADCC system answered both these problems. Reliability of the human ADCC assay has proven outstanding. It was after one year's experience with the ADCC assay that we were convinced of its reliability and sought to apply this assay to the water reuse project. In our hands the intra-assay variability of ADCC is 5%. Internal controls are available with each determination of the assay to assure its interpretability. Included are positive controls (cells, antibody, and target) and negative controls (cells, and target, antibody and target, and target alone). These controls have proven to be consistent.

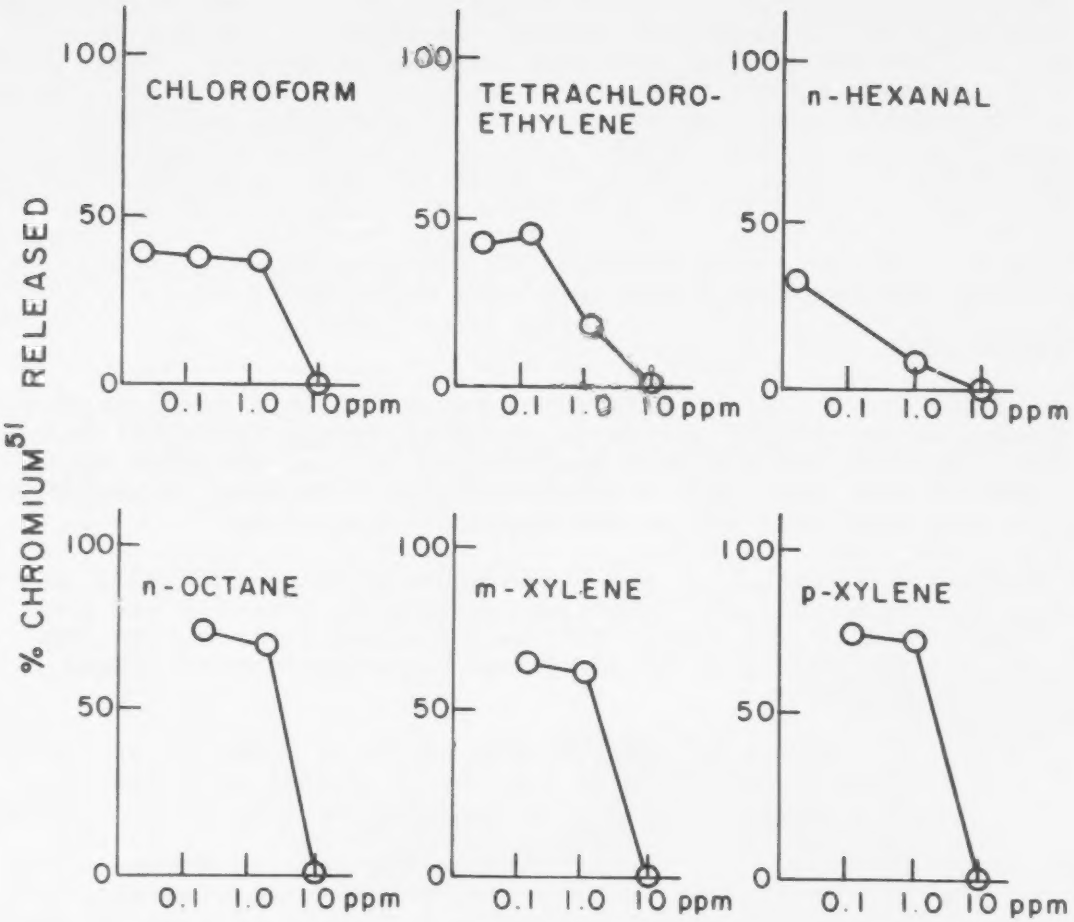

Figure 44. ADCC assay: killing power as a function of concentration of known toxic substances using monocytes. (continued)

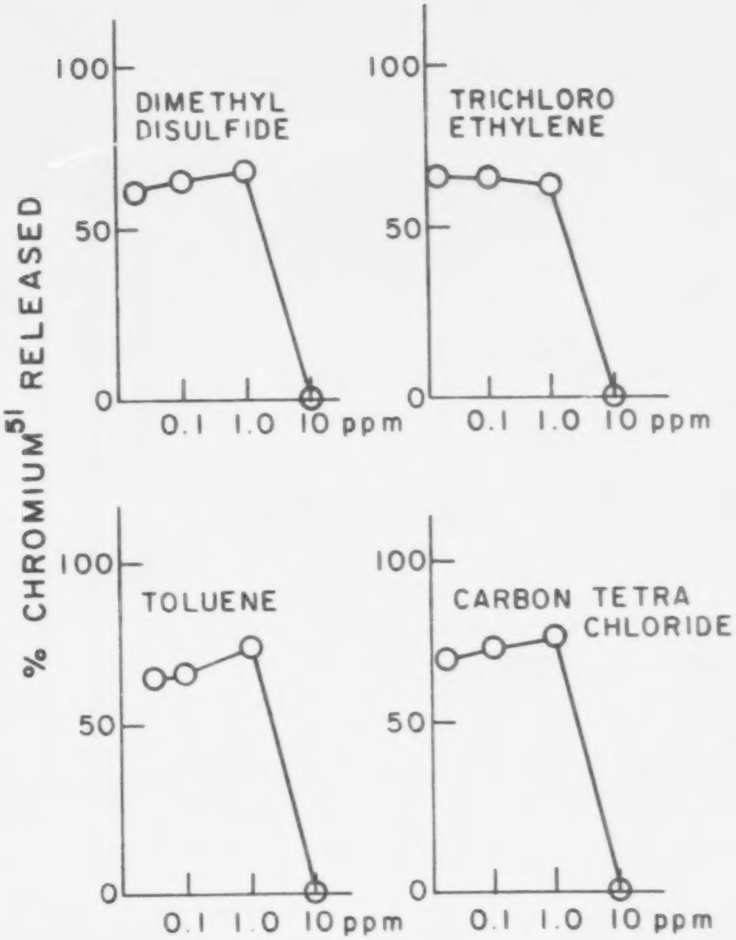

Figure 44 (continued). ADCC assay: killing power as a function of concentration of known toxic substances using monocytes.

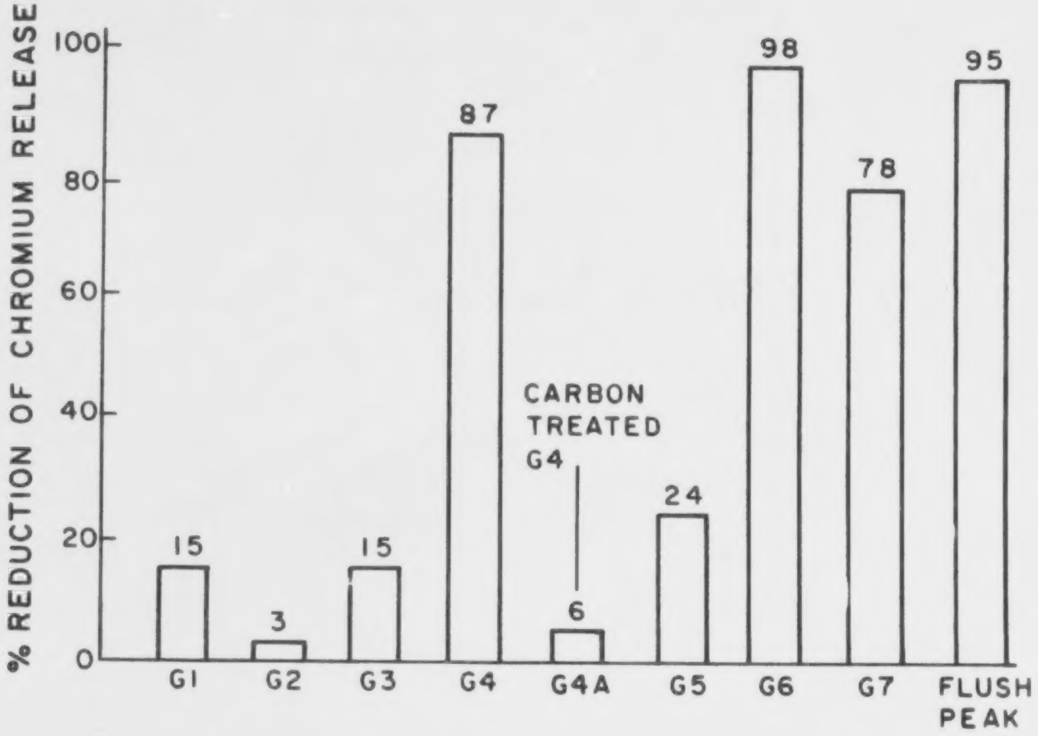

Figure 45. Comparative toxicities of fractions from Pomona, California wastewater by chromium release.

CONCLUSION

The conclusions from these studies indicate:

1) Neutrophil phagocytosis and killing power are not generally suitable for large scale toxicity studies of the type needed for on-line water monitoring.
2) Monocytes are more stable than neutrophils and large batches can be processed at one time. The depression in monocyte function induced by toxic compounds is, with few exceptions, most significantly observable in the concentration range 10 ppm and corresponds to metabolic changes observed. Relatively few compounds have any great effect on the monocyte function at the 0.1 and 1 ppm concentrations.
3) A wide range of responses was easily elicited by concentrated wastewater fractions and wastewater treatment steps were clearly detected by this assay.

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APPENDIX A

TESTING OF WASTEWATER FRACTIONS FOR THE PRESENCE OF POSSIBLE CARCINOGENIC SUBSTANCES EMPLOYING THE AMES SALMONELLA/MAMMALIAN MICROSOME MUTAGENCICITY TEST

by

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OBJECTIVE

The objective of this work was to supplement the toxicity testing of Dr. Solomons with a test for mutagenicity. While the time required for the Ames test precludes it from being part of an on-line, fast response system, there is a need to test for substances which might be carcinogenic. Thus far the Ames test is the fastest, least expensive, and most reliable in vitro test that gives information relating to carcinogenicity. Since there are indications of the existence of mutagens in the effluent from the Denver Metro Sewage Plant, it is of particular importance to monitor the effectiveness of the advanced treatment system in removing these mutagens. Concentrations and fractionation of the samples was carried out by Dr. Walton, and then the various fractions were tested by us, using the Ames Salmonella test, as described below.

GENERAL DESCRIPTION

The rational behind the Ames test is based on two major observations. First, about 90% of all carcinogens tested thus far are also mutagens (1). Second, mutagens react with and alter DNA, and DNA has the same double helical structure and the same four nucleotides in all living beings. Thus, it is quite reasonable to use bacteria (or any living organism) for the detection of potential mutagens for humans. Bacteria are easy to culture and the availability of well-characterized mutant strains make them a very attractive system to use for that purpose.

Ames and his collaborators have developed several bacterial tester strains containing different types of histidine mutations (2).

One strain (TA 1535) can be used to detect mutagens causing base-pair substitutions and two (TA 1537 and TA 1538) can be used to detect various kinds of frameshift mutagens. In addition to the histidine mutation, each tester strain contains two additional mutations that greatly increase its sensitivity to mutagens; one causes loss of the excision repair system and the other loss of the lipopolysaccharise barrier that coats the surface of the bacteria. The sensitivity of the test has been increased by introducing a resistant transfer factor (R factor) carrying a gene for resistance to ampicillin into the strains just described. This has made it possible to detect classes of carcinogens not previously detected (2).

The test can be performed in several ways, each with its advantages and limitations. The standard procedure is the plate incorporation assay. In this procedure an overnight culture of a bacterial tester strain is mixed with the sample to be tested (presumed mutagen) in molten agar at 45°C, and the mixture is poured on top of a plate containing minimal glucose agar. Only histidine-independent revertants can grow on this medium, and by counting the number of revertants a measure of the strength of the mutagen can be determined. Revertants are generally scored after two days of incubation. Many compounds are not mutagenic per se, but can give rise to mutagenic substances if acted upon by enzymes in a mammalian host (metabolic activation). To test for this possibility, a fraction of rat liver homogenate (S-9 fraction) can be added to the above mixture. This is the standard method that has been used for validating the test using hundreds of chemicals. For initial screening of a chemical testing, concentrations over a wide dose range (say 0,2, 20 and 500 μg per plate) are recommended both in the presence and absence of the standard S-9 mix. A positive or questionable result should then be confirmed by demonstrating a dose response effect using a narrower range of concentrations. In general, Ames and co-workers find that for most mutagens a concentration range exists when there is a linear dose response relation, and the revertants per plate reported for any mutagen should be taken from this region of the curve. In each experiment positive mutagenesis controls are routinely included using diagnostic mutagens to confirm the reversion properties of each strain.

A very useful variation is the use of spot tests. Spot tests are the simplest way to test compounds for mutagenicity and are particularly adaptable for the initial rapid screening of large numbers of compounds in a short period of time. There are several advantages to the spot test and it is often useful to test all new compounds by this method before doing the standard plate incorporation test. No solutions are necessary since a few crystals (or μℓ of liquid) can be put directly on the agar surface; also, since the compound diffuses out from the central spot, a range of concentrations are tested simultaneously. The spot test affords a preliminary indication of the toxicity of the chemical for the bacteria by the size of the zone of inhibition of the background lawn of bacterial growth around the spot; it further shows whether or not the S-9 mix is necessary for mutagenicity, and in the case of a positive result, indicates which tester strain should be used for the dose response curve. The spot test is primarily a qualitative test, and although very useful, has distinct limitations. It can only be used for the detection of chemicals which are diffusable in the agar, and thus most polycyclic hydrocarbons and other water insoluble chemicals are not easily detected by this procedure. It is also much less sensitive than the standard plate test as only relatively few bacteria on the plate are exposed to the chemical at any particular dose level.

By the use of this test about 300 carcinogens and non-carcinogens have been tested for mutagenicity (1) and a high correlation between carcinogenicity and mutagenicity demonstrated; ninety percent (156/174) of carcinogens are also mutagens while few non-carcinogens show any degree of mutagenicity. The carcinogens comprise a wide variety of chemical types including alkylating agents, nitrosamines, polycyclic hydrocarbons, fungal toxins, aromatic amines, nitrofuran carcinogens, a variety of neoplastic agents, and antibiotic carcinogens such as adriamycin, daunomycin, and mitomycin C. Also, known human chemical carcinogens which have been tested are positive. These include B-naphthylamine, benzidine, cigarette smoke condensates, bis-chloromethylether, aflatoxin B_1, vinyl chloride, 4-aminobiphenyl, etc (1).

The Ames test can be used to test not only pure compounds but complex mixtures as well, and thus has a very wide range of applications. Because linear dose response curves are usually observed the test is quite valuable as a bio-assay in identifying and purifying mutagenic components in complex mixtures. For example, it has been useful in determining the mutagenic activity of cigarette smoke condensates and 12 standard smoke condensate fractions (3), in commercial hair dyes (4), and soot from city air (mentioned in 1). Other applications of the salmonella mutagenesis test include the detection of mutagenic metabolites in urine (2). This would be extremely useful in analyses of human urine from individuals who may be heavily exposed to presumptive carcinogens.

In summary, the Ames test is ideally suited for rapid testing of environmental pollutants to determine their mutagenic and carcinogenic potential. Positive results in this test would clearly indicate that the chemical (or chemicals) in question represent a potential human health hazard and should be thoroughly tested in animal systems; where extensive human exposure has occurred, appropriate epidemiological studies should be performed.

To test compounds on unknown mutagenicity we use the following protocol:

1) Spot tests on all tester strains using the highest possible concentrations of compound or mixture being tested.
2) If results are negative, perform full plate tests (plate incorporation assays) on all tester strains again using the highest possible concentration of compound. This is to see whether the compound has weak mutagenic activity and this is difficult to ascertain in a spot test.
3) If results of initial spot tests are positive, a dose response study employing the tester strain giving the best response will be carried out.

We tested nine fractions of Denver wastewater (samples G1 through G9) supplied by Dr. Walton. Approximately eight liters of secondary sewage effluent were preconcentrated by fractional freezing, then processed in the manner outlined in the text, ending with 3 mℓ of each of the fractions. Full plate tests were conducted in duplicate with each strain, both with and without S9 (microsomal rat liver fraction). In each case we added 0.3 mℓ of each sample (undiluted) per plate. The following controls were routinely carried:

1) Checked tester strains for normal response to mutagens by using a standard set of known chemical mutagenic agents such as nitrosoguanidine, 2-aminofluorene and daunomycin.
2) Possible effect of methanol (which was the solvent employed) on the observed results was tested by running a set of control plates to which methanol was added to the bacteria.
3) Standard set of control plates containing only bacteria (with and without S9) was used to determine spontaneous reversion frequency.

The results of these tests are shown in Table A-1, which gives the average number of revertants per plate. According to the guidelines set up by Ames and his collaborators, an increase in the number of induced revertants less than two-fold over the spontaneous number was not significant. The results of these tests clearly indicated that mutagenic substances were present in samples G1, G2, and G3. While we found no indication of mutagenic activity in samples G4 through G9, this should not be interpreted to mean that these samples were free of potential mutagens. It is possible that mutagens were present, but that their concentrations were too low to be detected by our assay.

TABLE A-1. RESULTS OF FULL PLATE AMES TESTS ON DENVER WASTEWATER FRACTIONS

Strain	S9	Control	G1	G2	G3
TA 1538	+	34	299	162	371
	-	31	46	60	32
TA 98	+	72	450	108	238
	-	28	15	101 (?)	90
TA 1537	+	16	100	475	171
	-	15	106	138	91
TA 1535	+	33	742	run out	run out
	-	18	35	run out	run out
TA 100	+	48	285	run out	run out
	-	15	30	run out	run out

The mutagenic activity displayed by the first set of samples was important enough to warrant further confirmatory tests. Consequently, Dr. Walton processed more secondary effluent, taking 8 liters each of both chlorinated and unchlorinated wastewater. As before, each sample was preconcentrated by freezing and fractionation, and we were supplied with 12 concentrated fractions of chlorinated and unchlorinated effluent. This time we used .1 and .2 mℓ of each sample.

As shown by Table A-2, there was no positive mutagenic response; values for both chlorinated and unchlorinated samples are within an acceptable range of the controls.

CONCLUSIONS

The difference between the mutagenic activity of the two sets of samples is most probably due to the difference in the times of collection of the effluent. This would indicate that the concentrations of compounds and mutagens, as well as the types of compounds, vary underlining the necessity for routine mutagenicity testing.

TABLE A-2. RESULTS OF CONFIRMATORY AMES TESTS ON DENVER WASTEWATER FRACTIONS

Strain	S9	Sample	Control	G1	G2	G3
TA 1538	+	chlorinated	25	44	26	30
		unchlorinated	12	39	33	55
	-	chlorinated	15	12	28	28
		unchlorinated	4	40	18	13
TA 98	+	chlorinated	65	36	62	56
		unchlorinated	37	78	68	70
	-	chlorinated	34	53	52	61
		unchlorinated	8	42	51	56
TA 1537	+	chlorinated	10	10	14	10
		unchlorinated	12	34	32	26
	-	chlorinated	13	21	5	15
		unchlorinated	19	23	17	33
TA 1535	+	chlorinated	29	28	26	35
		unchlorinated	20	15	11	21
	-	chlorinated	28	26	29	23
		unchlorinated	22	26	22	20
TA 100	+	chlorinated	17	16	30	23
		unchlorinated	24	21	20	22
	-	chlorinated	30	31	31	25
		unchlorinated	28	27	20	22

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APPENDIX B

USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN LAYER CHROMATOGRAPHY IN THE RAPID DETECTION OF HUMAN CELLULAR TOXICITY OF ENVIRONMENTAL SUBSTANCES

by

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ABSTRACT

The amount of ATP (adenosine Triphosphate) and the rate at which it can be synthesized is important in maintaining adequate cellular functioning. Consequently, any interference in the storage and production of purine nucleotides can be regarded as potentially toxic.

A series of pure organic compounds and fractions isolated from treated sewage effluent and water treatment plant was placed in contact with human neutrophils and platelets. Trace amounts of U-14-C adenine were added to the cell suspensions which were incubated at 37°C for 30 minutes. Perchloric acid extracts were analyzed by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) for ATP, ADP, AMP, uric acid, hypoxanthine, and xanthine. Dilutions of the compounds were used to obtain dose response curves. The HPLC methodology is capable of automation and could perform a 5-point dose response curve every hour. Factors involved in the utilization of these techniques for the on-line surveillance of renovated water for human consumption are discussed.

INTRODUCTION

Stress may be regarded as any influence which causes the cell to increase the work it must do to maintain itself and carry out its function. Stress effects are largely reversible when held within physiological limits. However, significant increases in cellular entropy content due to interference with information processing or membrane stability within the cell can lead to permanent pathological damage expressed in a variety of ways depending upon which structures and metabolic pathways are specifically affected. Serious stress effects include mutation, transformation to malignant states, loss of energetic capability for ion pumping, and cell death followed by lysis, which can yield further toxic breakdown products.

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*Presented at the 12th Annual Conference on Trace Substances in Environmental Health, June 5-8, 1978, University of Missouri, Columbia, Mo.

The ultimate source of energy for all cellular functioning is the ATP (adenosine triphosphate) molecule synthesized by oxidative enzymatic catalysis involving subcellular mitochondrial membranes and cytoplasmic anaerobic glycolsis. ATP derives its energy ultimately from the sun via edible fuels such as carbohydrates and protein and is a universally acceptable mediator of all cell work.

Consequently, a disturbed ATP turnover of concentration is a serious sign of impending damage to the cell. Degradation of ATP can form several products including hypoxanthine (HYPX), which is salvable to some extent to reform ATP (Figure B-1). However, the appearance of large amounts of HYPX together with a reduced pool of ATP is a sign of severe cellular stress.

The above rationale has been used to develop toxicity detection criteria for the testing of a wide variety of compounds in drinking water, and is capable of automation and on-line operation. A series of cells or subcellular organelles from animal, plant or other biological origin can be used to evaluate the environmental impact of a trace contaminant. Water can be used directly, after adjustment of osmotic pressure, or the water can be concentrated and fractionated and each fraction separately evaluated. A 5-point dose response curve can be constructed every two hours and the results evaluated by computer to signal an alarm according to present criteria. The remainder of this paper discusses the methodology and results obtained using pure compounds and concentrated water fractions.

MATERIALS AND METHODS

Cells

Neutrophils and monocytes supplied by Dr. Weston and platelet-rich-plasma (1) which we prepared by standard methods were presented for analysis after exposure to four or five concentration levels in the range 0-10 ppm of the test compound. Fractions obtained by Dr. Walton from sewage and water treatment plants were also tested. Five microliters of U-14-C adenine (60,000 cmp) were added to a 0.5-milliliter suspension of cells 5000-10,000 cu mm. After incubation at $37^{o}C$ for 30 minutes the cells were cooled in ice and centrifuged at 3000 rpm for 3 minutes. The supernatant was removed and 0.2 mℓ of cold 13% perchloric acid (PCA) was added to the cell pellet. After mixing

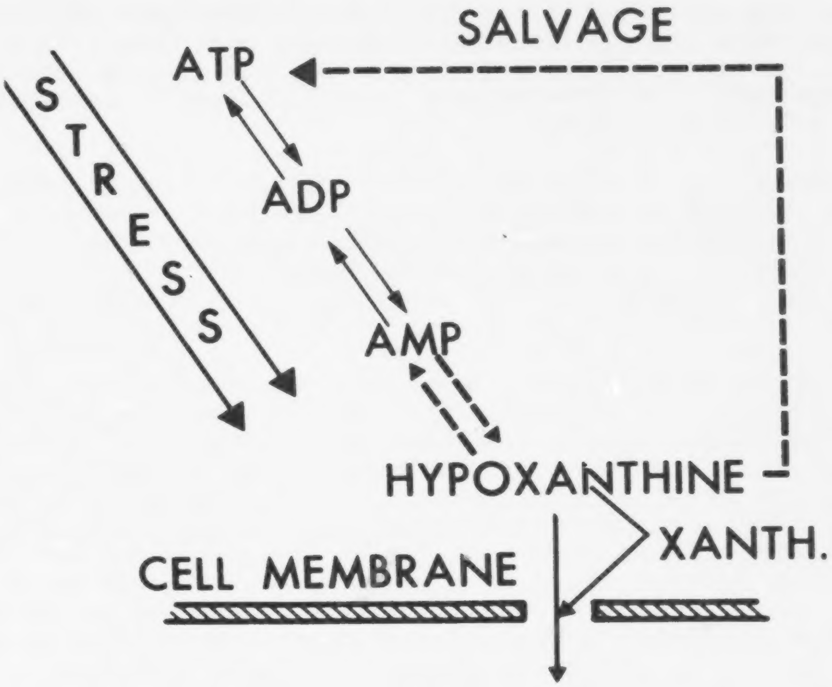
SALVAGE
ATP
STRESS
ADP
AMP
HYPOXANTHINE
XANTH.
CELL MEMBRANE

Figure B-1. Effect of stress on ATP metabolism.

and centrifuging, the PCA supernatant was applied to the chromatographic systems described below.

Chromatography

High Performance Liquid Chromatography (HPLC)--

A Waters & Associates HPLC apparatus with C_{18}-Bondapak reversed phase column was used. Fifteen microliters of PCA extract was injected and 0.1M KH_2PO_4 was pumped through the column at 1.5 mℓ/min. The nucleotides and related compounds were separated in 12-15 minutes and were detected by UV absorption at 254 nm. Identification was obtained using retention time and the addition of known compounds to the test PCA supernatant. The effluent from the HPLC system can be pumped through a flow-through scintillation spectrometer so that the specific activity of each compound could be computed. In these experiments a suitable flow-through spectrometer was not available; the effluent was collected into twenty fractions and counted in a scintillation spectrometer. Recovery and reproducibility of the standards was 95-103%. The peak height was found to be linearly proportioned to concentration, regardless of peak width, in the range of 10^{-9} to 10^{-11} moles of purine compound per sample injected (Figure B-2).

Thin Layer Chromatography (TLC)--

Ten microliters of the PCA supernatant were applied without heat to a 2-centimeter strip of an Eastman Kodak Cellulose Thin Layer Chromatogram #13255 with a mylar backing, and developed with a mixture of water: formic acid: tertamyl alcohol: 1:2:3 for 5-6 hours (2). The chromatogram was dried in air, cut into 15, 1-centimeter strips, and placed in a 5-milliliter counting vial with 3 mℓ of scintillation fluid. The vials were counted in a scintillation spectrometer and each count calculated as a percentage of the total count.

RESULTS

Adequate and rapid chromatographic separation of the purine nucleotides and their metabolites was obtained by HPLC (Figure B-2). The relationship between peak height and concentration was linear throughout. This method is capable of detecting 1×10^{-11} moles of purine compound in the intracellular pool. Some raw data is shown in Figure B-3. The TLC separations were not as sharp but did provide essential data on the rate of ATP synthesis on the order of 10^{-10} moles/hr, independent of the pool size (Figure B-4). Table B-1 lists the response of neutrophils and platelets to various pure compounds and to fractions obtained from drinking water. As an example, the dose response curve for chlorofrom is plotted in Figure B-5.

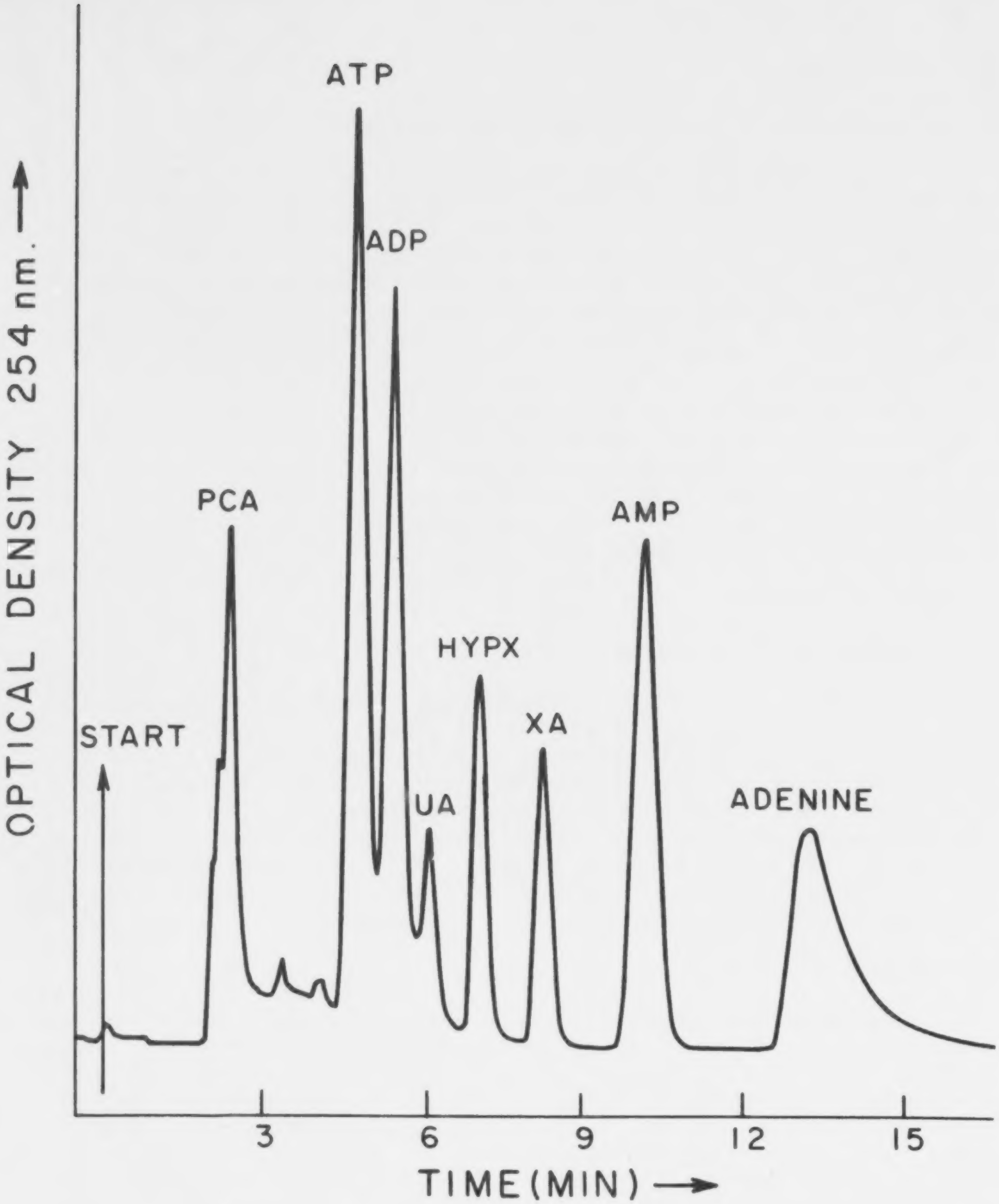

Figure B-2. Elution curve of pure standards using HPLC.

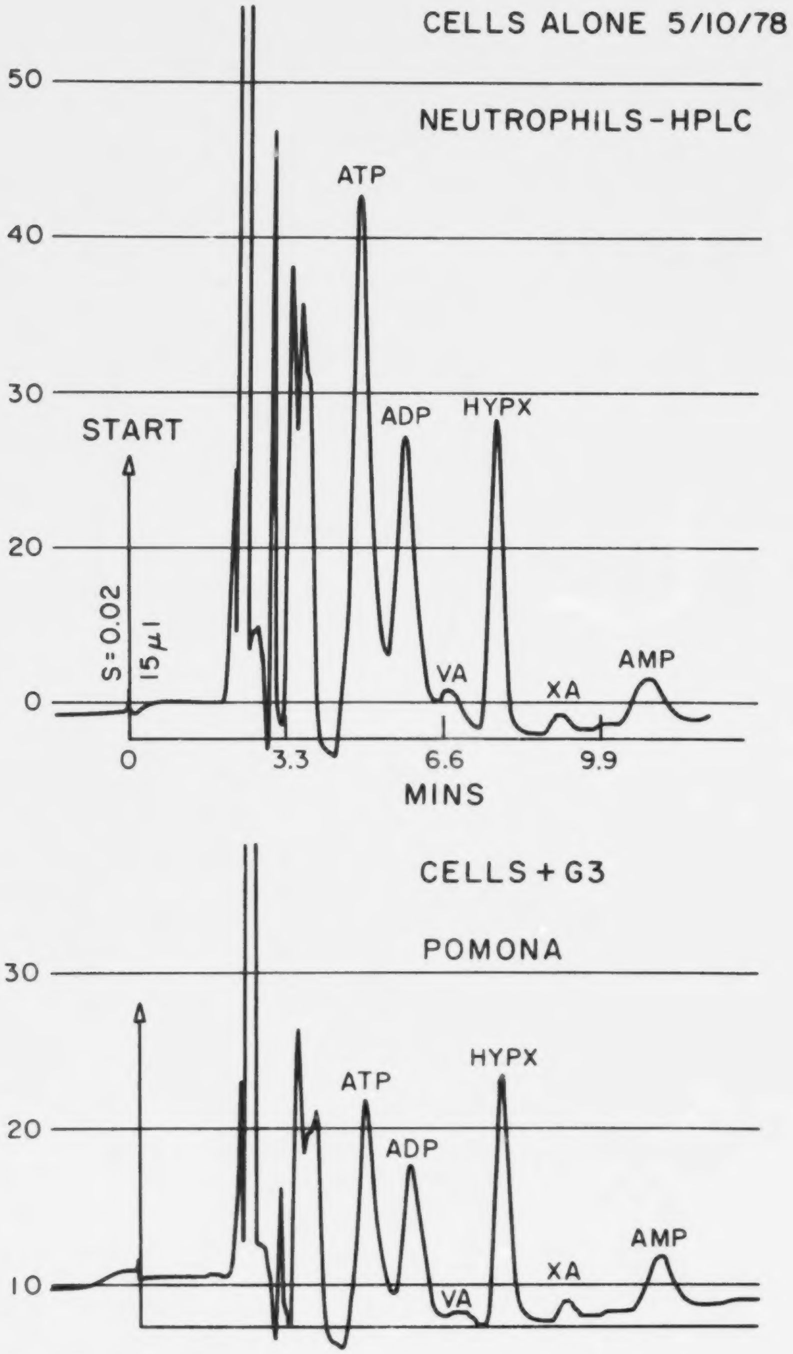

Figure B-3. HPLC raw data.

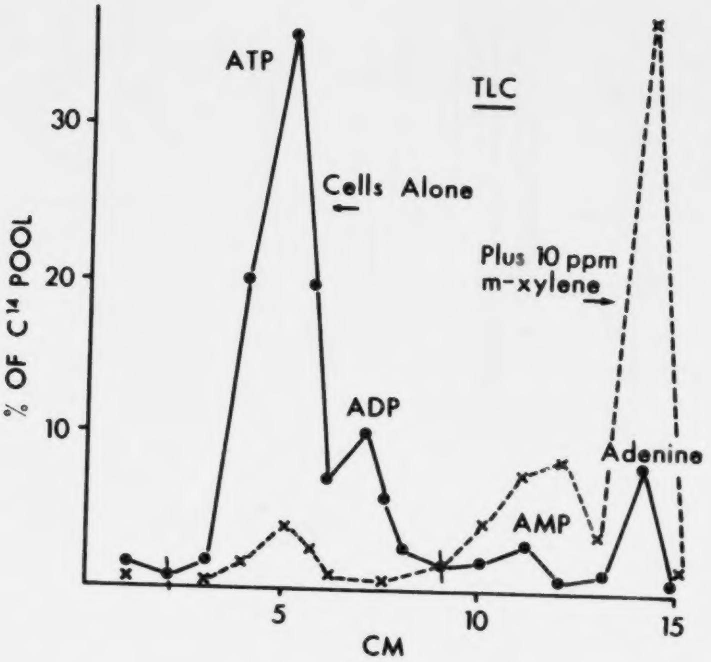

Figure B-4. Toxic effect of m-xylene on platelets using TLC.

TABLE B1. RESPONSE OF NEUTROPHILS AND PLATELETS TO VARIOUS PURE COMPOUNDS AND TO FRACTIONS OBTAINED FROM DRINKING WATER

	Added conc. ppm	% $C^{14}\overset{\text{TLC}}{\overline{\text{ATP}}}$ of C^{14} pool	$C^{12}\overset{\text{HPLC}}{\overline{+\,C^{14}}}$ ATP peak ht cm	Cell
Hexanol	0.0	50.0	-	Neutrophil
	0.1	4.1	-	
	1.0	0.0	-	
	10.0	0.0	-	
Trichloroethylene	0.0	62.0	11.0	Neutrophil
	0.01	0.0	4.2	
	0.10	0.0	2.1	
	1.00	0.0	3.5	
	10.00	0.0	1.4	
Toluene	0.0	61.0	10.9	Neutrophil
	0.1	63.0	12.2	
	1.0	66.0	12.6	
	10.0	5.3	0.4	
Metaxylene	0.0	57.0	8.6	Platelet
	0.1	63.0	9.6	
	1.0	61.0	8.5	
	10.0	6.2	0.0	
Chloroform	0.0	59.0	4.5	Neutrophil
	0.1	60.0	4.5	
	1.0	45.0	2.7	
	1.3	28.0	-	
	2.6	17.0	-	
	10.0	2.9	0.7	

(continued)

TABLE B1 (continued)

	Added conc. ppm	TLC % $\frac{C^{14}\ ATP}{C^{14}\ pool}$ of	HPLC $C^{12} + C^{14}$ ATP peak ht cm	Cell
Napthalene 2-methoxy, 6-isoproprionic acid	0.0	-	23.0	Platelet
	0.1	-	21.4	
	1.0	77.0	21.8	
	10.0	79.0	20.8	
2-bromo 6 methoxy napthalene	0.0	-	23.0	Platelet
	0.1	63.0	24.5	
	1.0	74.0	17.9	
	10.0	61.0	16.0	
2-methoxy-naphthalene	0.0	-	23.0	Platelet
	0.1	76.0	21.6	
	1.0	68.0	22.7	
	10.0	74.0	20.5	
n-octane	0.0	72.0	-	Neutrophil
	20.0	63.0	-	
n-hexane	0.0	72.0	-	Neutrophil
	20.0	25.0	-	
111-trichloroethane	0.00	70.0	-	Neutrophil
	0.10	17.0	-	
	1.00	13.0	-	
Dichlorobenzene	0.00	71.0	-	Neutrophil
	0.01	68.0	-	
	0.10	66.0	-	
	1.00	72.0	-	

(continued)

TABLE B1 (continued)

		TLC	HPLC	
	Added conc. ppm	% C^{14} ATP of C^{14} pool	C^{12} + C^{14} ATP peak ht cm	Cell
Cincinnati drinking water concentrate (supplied by Dr. McCabe)	0.00	76.0	17.2	Platelet
	1.40	62.0	15.3	
	1.40	-	11.7	
Fraction G4 Secondary Effluent Pomona	0.0	51.0	10.0	Neutrophil
	1.1	5.8	0.0	
Negative Controls				
(i) Dimethyl sulfide	0.0	55.0	11.0	Platelet
	12.5	53.0	10.6	
	25.0	45.0	10.3	
(ii) Glucose	0.0	56.0	13.0	Platelet
	600.0	54.0	13.6	
	1200.0	56.0	13.5	

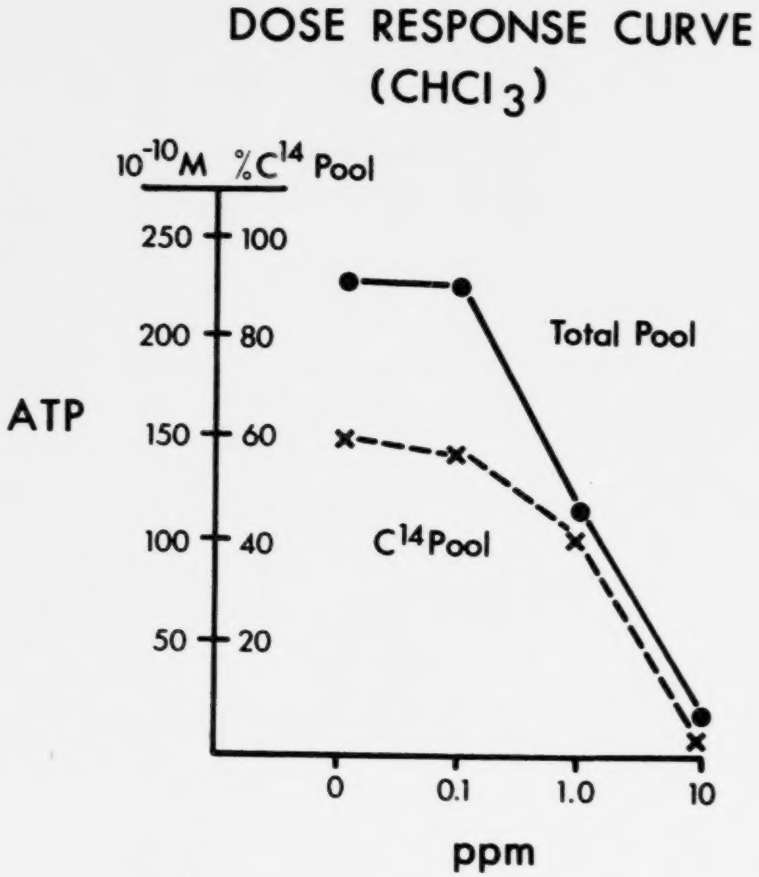

Figure B-5. Dose response curve for chloroform.

DISCUSSION

The utilization of cell incubation followed by HPLC and TLC analysis offers a considerable capability for the sensitive and rapid detection of cellular toxicity. The system is flexible in that it is not limited to any cell type or environmental substance, and is relatively inexpensive. The system is vulnerable in that it needs a constant supply of normal cells or organisms. However, continuous 5-point dose response curves can be obtained using platelets from 10 mℓ of blood per day available from a nearby blood bank. Alternatively, one could use in vitro cell cultures of fibroblasts or bacteria, which can be made in large batches. As long as sufficient cells are present an accurate cell count is not needed, provided the same suspension is used within 5 hours for test and control. If necessary, a portion of the cells can be diverted for functional studies or other confirmatory investigations. The test substance is usually in solution, but this is not an absolute requirement as the toxic effects of exposing platelets to different surfaces can also be determined (Figure B-6). The monitoring is capable of continuous on-line operation as indicated in Figure B-7. Although this work was concerned with ATP because of the universality of its function in living systems, one is not restricted to this pathway. The same approach can be used to focus on a variety of metabolic and enzymatic effects and any cellular function which is of interest.

The monitoring can also be done to study internal unit processes such as reverse osmosis permeate and reject in an advanced water treatment plant. Thus, procedures such as reverse osmosis and activated carbon treatment, etc., can be evaluated from the biological standpoint with reference to plant, animal, and human cells.

Extrapolation from these results to gross human toxicity data available in RTECS shows that certain correlations exist. For instance, chloroform is more toxic than toluene in both the cellular ATP response and LD50 dosages. Although there are many factors which influence the toxic effects of a chemical, a broad correlation is present, indicating that cellular ATP measurements are not too far removed from clinical toxicity effects. These measurements, in fact, offer us a more meaningful method of evaluation in many cases, especially when exposure levels are low. The results on platelets are in accordance with previous work by other investigators on blood cells and biomaterials (3).

CONCLUSION

It is concluded that HPLC chromatography of cellular purines may offer a sensitive, economic, and biologically significant method of detecting toxicity. The system is worthy of

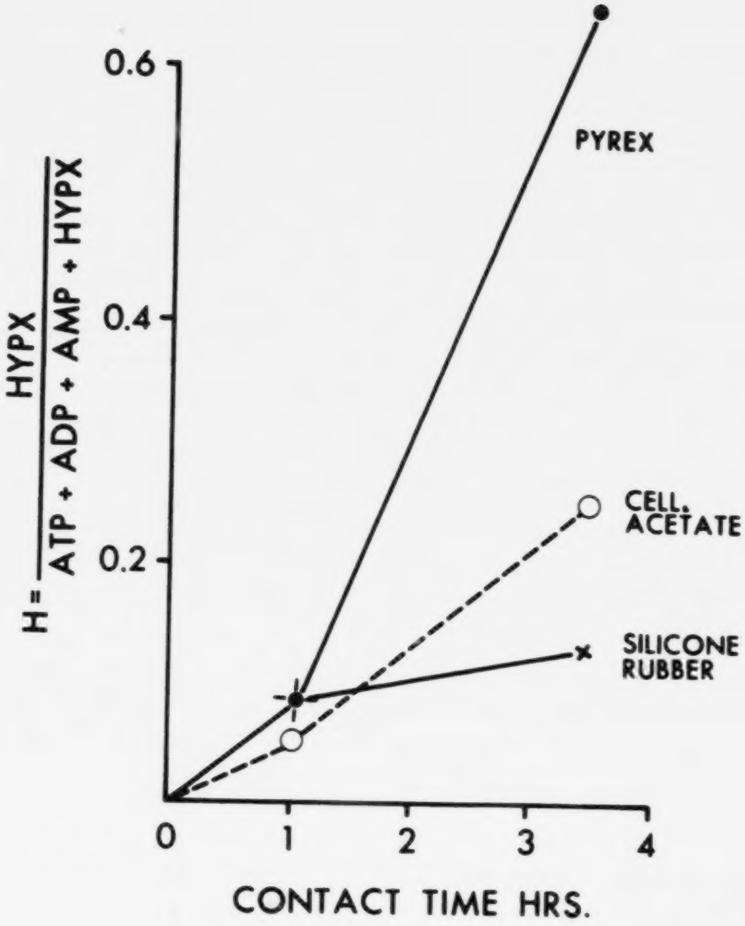

Figure B-6. Effect of foreign surfaces on nucleotide metabolism. H= irreversible loss of purines from the platelets.

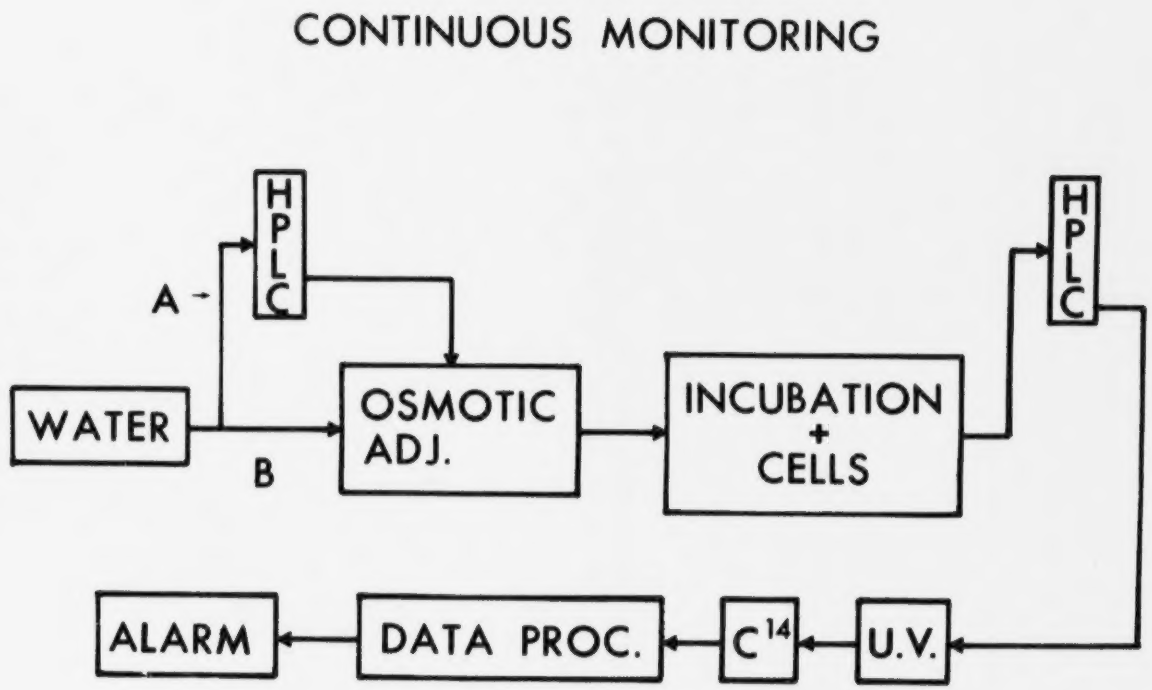
CONTINUOUS MONITORING
WATER
A
B
HPLC
OSMOTIC ADJ.
INCUBATION + CELLS
HPLC
U.V.
C14
DATA PROC.
ALARM

Figure B-7. Proposed on-line monitoring system.

consideration for the automated testing of water-borne environmental substances.

ACKNOWLEDGMENTS

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